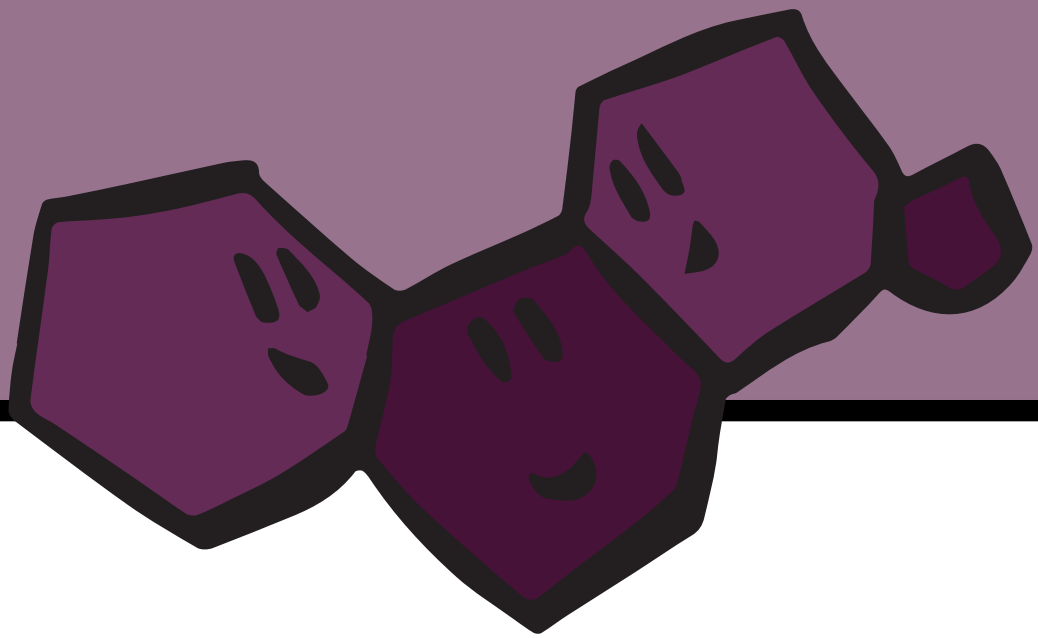
Congenital adrenal hyperplasia

about causes and consequences



Manon Engels

Stellingen behorende bij het proefschrift

Congenital adrenal hyperplasia

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1. Ook bijnier steroïd precursors hebben glucocorticoïde werking en kunnen daarmee gedeeltelijk compenseren voor cortisoldeficiëntie aanwezig in patiënten met adrenogenitaal syndroom. *(dit proefschrift)*

2. De definitie van cortisoldeficiëntie moet opnieuw ter discussie gesteld worden. (*dit proefschrift*)

3. Testiculaire bijnier resttumoren zijn een veel voorkomende langetermijncomplicatie en de belangrijkste oorzaak voor een verminderde gonadale functie in mannen met adrenogenitaal syndroom. (*dit proefschrift)*

4. Testiculaire bijnier resttumoren hebben zowel bijnier als testiculaire eigenschappen en ontstaan uit een pluripotente cel door stimulatie met ACTH. (*dit proefschrift)*

5. Op basis van kwaliteit van leven gaat het erg goed met mannen met adrenogenitaal syndroom. (*dit proefschrift)*

6. Ik heb er wèl de ballen verstand van.

Manon Engels
Nijmegen, 18 december 2018

Congenital adrenal hyperplasia

about causes and consequences

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The research presented in this thesis was performed at the department of Pediatrics and the department of Laboratory Medicine, Radboud Amalia Children's hospital and Radboud Institute for Molecular Life Sciences, Radboud university medical center, the Netherlands.

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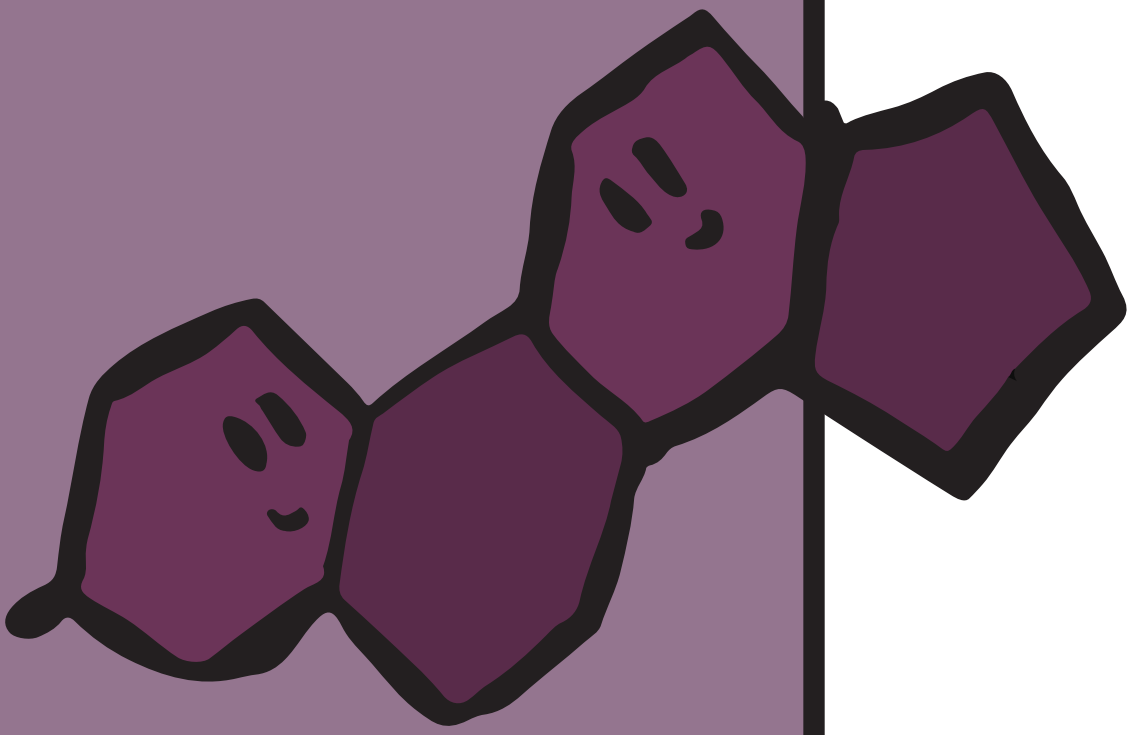

Chapter 1

General introduction

The adrenal gland

The human body contains two adrenal glands, located superior to the kidneys. Each gland has a cortex and a medulla (Fig. 1.1A). Embryologically, the medulla originates from neural crest tissue, while the adrenal cortex arises from the coelomic mesoderm of the urogenital ridge of which also the kidneys and gonads are formed. The adrenal glands have important endocrine functions. Chromaffin cells in the medulla are able to produce epinephrine and norepinephrine (also called (nor)adrenalin(e)), while the cortex produces steroid hormones. The adrenal cortex is divided into three zones with different steroid synthesis characteristics: the zona glomerulosa, the zona fasciculata and the zona reticularis (Fig. 1.1A). Adrenal steroidogenesis converts cholesterol via several enzymatic steps to three types of steroid hormones: mineralocorticoids, glucocorticoids and androgens (Fig. 1.1B). Expression of adrenocortical enzymes differs between the zones, but all zones express cholesterol side-chain cleavage enzyme and 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-hydroxysteroid dehydrogenase), two common enzymes of all steroidogenic cells. Furthermore, the zona glomerulosa expresses 21-hydroxylase, cytochrome P450 11B1 (11-hydroxylase) and cytochrome P450 11B2, but not 17α-hydroxylase/17,20lyase (17-hydroxylase), resulting in the production of mineralocorticoids. Mineralocorticoids, such as aldosterone, are responsible for the regulation of blood pressure and electrolyte balance and its secretion is regulated by the renin-angiotensin-aldosterone system. The zona fasciculata expresses 17-hydroxylase, 21-hydroxylase, and 11-hydroxylase resulting in the production of glucocorticoids. Glucocorticoids, such as cortisol, regulate glucose metabolism and immune response. The production of cortisol is regulated by the hypothalamus-pituitary-adrenal (HPA) axis. The hypothalamus produces corticotrophin-releasing hormone (CRH), which stimulates the pituitary gland to produce adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal gland to produce cortisol. Cortisol functions in a negative feedback loop as it inhibits CRH and ACTH synthesis. In contrast to the other zones, the zona reticularis does not express 21-hydroxylase and 11-hydroxylase, but does express 17-hydroxylase resulting in production of adrenal androgens, such as dehydroepiandrosterone (DHEA) and androstenedione. Sulfation of DHEA leads to the inactive steroid DHEA-S.

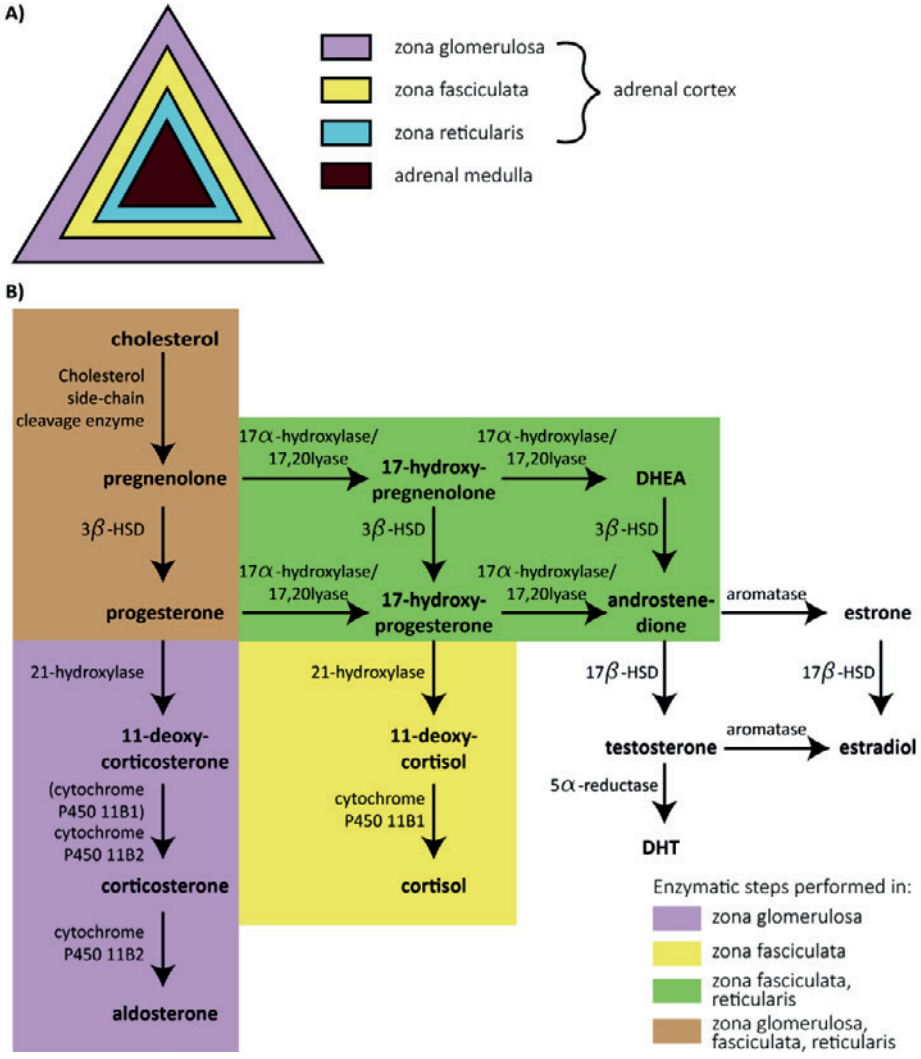

Fig. 1.1: The adrenal gland and adrenal steroidogenesis. A) Schematic representation of the adrenal gland consisting of a medulla and a cortex with 3 zones: zona glomerulosa, zona fasciculata, and zona reticularis. **B)** Adrenal steroidogenesis converting cholesterol to cortisol, aldosterone and adrenal androgens via several enzymatic steps in the adrenal cortex. Brown indicates enzymatic steps that are performed in all adrenal cortex zones, while green indicates enzymatic steps specific for the zona fasciculata and zona reticularis. The purple box indicates specific enzymatic steps for the zona glomerulosa, while within the yellow box enzymatic steps specific for the zona fasciculata are shown. In white, enzymatic steps converting adrenal androgens to sex steroids in peripheral tissues (mainly in the gonads) are presented. Abbreviations and synonyms: 17β-HSD, testosterone or estradiol 17β-dehydrogenase 3; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase; 5α-reductase, 3-oxo-5α-steroid 4-dehydrogenase 1; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone.

Congenital adrenal hyperplasia

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of the adrenal cortex caused by a defect of one of the enzymes involved in the adrenal steroidogenesis. The most common enzyme deficiency (>90%) causing CAH is 21-hydroxylase deficiency (*CYP21A2* mutation) with an incidence of 1:12 000 (1, 2). About 5% is caused by 11-hydroxylase deficiency (*CYP11B1* mutation). In this thesis we focus on patients with 21-hydroxylase deficiency.

Patients with CAH caused by 21-hydroxylase deficiency (Fig. 1.2A) suffer from impaired cortisol production and consequently elevated ACTH concentrations due to the lack of the negative feedback on the pituitary gland. The increased ACTH concentrations eventually lead to hyperplasia of the adrenal cortex and accumulation of steroid precursors before the enzymatic block (mostly 17-hydroxyprogesterone). These steroids are subsequently shunted to the unaffected androgen pathway and alternative pathways producing elevated concentrations of 21-deoxycortisol, 11-hydroxy-progesterone, 16-hydroxyprogesterone, 11-oxygenated androgens, and dihydro-testosterone. The severity of the disease depends on the residual 21-hydroxylase activity. The most severe patients have <1% residual enzyme activity with cortisol and aldosterone deficiency and are classified as group null, A, or classic salt-wasting type. Patients with 1-5% residual enzyme activity have cortisol deficiency, but sufficient mineralocorticoids and are classified as group B or classic simple virilizing type (1-3).

Patients with a very mild mutation in the *CYP21A2* gene have a residual enzyme activity of 20-50% and are classified as non-classic CAH. Non-classic CAH patients have generally no cortisol and aldosterone deficiency, but about two-third of the patients have suboptimal response to ACTH (4). Patients with non-classic CAH also have slightly elevated androgen concentrations, eventually resulting in clinical complaints related to hyperandrogenism mostly in girls, such as precocious puberty, irregular menses, acne, and hirsutism (5).

Other forms of CAH

The second most common enzyme deficiency (about 5%) causing CAH is caused by mutations in the *CYP11B1* gene, resulting in 11-hydroxylase deficiency (Fig. 1.2B). These patients also have impaired cortisol production and high ACTH and androgen concentrations. Patients with 11-hydroxylase deficiency suffer from hypertension, as increased concentrations of the adrenal steroid precursor 11-deoxycorticosterone, which has mineralocorticoid potency, are present (6).

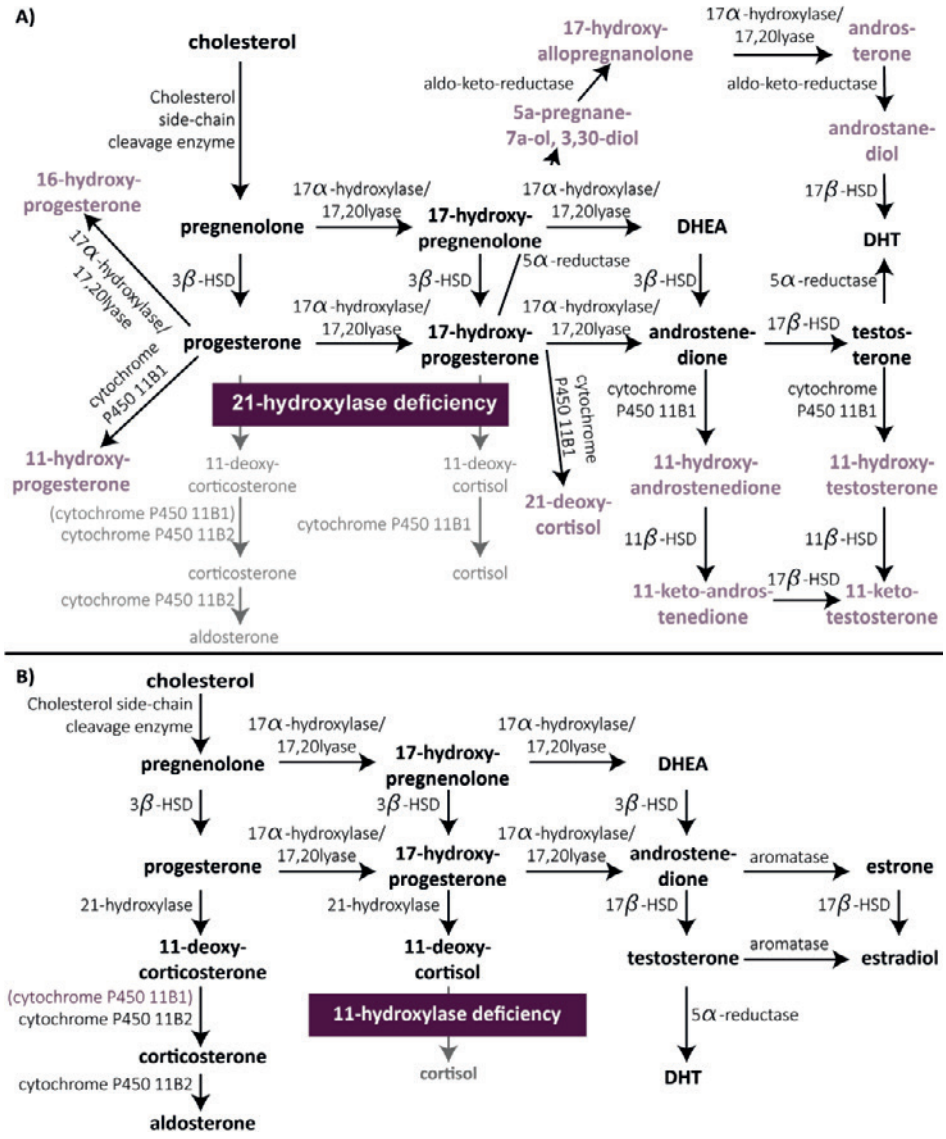

Fig. 1.2: The most common enzyme deficiencies of the adrenal cortex resulting in congenital adrenal hyperplasia. A) 21-hydroxylase deficiency causes impaired production of cortisol and aldosterone (indicated in gray), while adrenal steroid precursors (mostly 17-hydroxyprogesterone) before the enzymatic block accumulate. These steroid precursors are converted via several alternative enzymatic steps indicated in purple. **B)** 11-hydroxylase deficiency causes impaired cortisol (indicated in gray) and elevated adrenal androgen concentrations. Adrenal steroid precursors 11-deoxycorticosterone and 11-deoxycortisol accumulate. Abbreviations and synonyms: 11β-HSD, corticosteroid 11β-dehydrogenase; 17β-HSD, testosterone or estradiol 17β-dehydrogenase 3; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase; 5α-reductase, 3-oxo-5α-steroid 4-dehydrogenase 1; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone.

Mutations in *HSD3B2*, *CYP17A1*, *POR*, *StAR*, and *CYP11A1* are rare, but can also cause CAH (2, 7). All have in common the impaired production of cortisol leading to increased ACTH concentrations, but with different deficiencies or accumulation of steroid precursors based on the type of defect. For example, patients with 17-hydroxylase deficiency also have low peripheral aldosterone and androgen concentrations, but exhibit high concentrations of 11-deoxycorticosterone and corticosterone, which cause hypertension (8). 3β-hydroxysteroid dehydrogenase deficiency also results in low aldosterone and androstenedione concentrations, but increased concentrations of the precursor steroid DHEA (9).

Diagnosis of CAH

In CAH patients, adrenal steroid precursors before the enzymatic block will accumulate. One of the hallmarks of patients with 21-hydroxylase is therefore an increased concentration of the adrenal steroid precursor 17-hydroxyprogesterone, and diagnosis of 21-hydroxylase is therefore based on increased concentrations of this steroid. Measurement of 17-hydroxyprogesterone in dried blood spots is included in the Dutch neonatal screening program. Confirmatory testing using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is indicated, especially when primary measurement was performed with immunoassays (1). Nowadays, measurement of 21-deoxycortisol is also suggested to diagnose 21-hydroxylase deficiency, as steroid production of 21-deoxycortisol is negligible in healthy persons (10). Preferably, mutation analysis should also be performed to confirm the diagnosis of CAH. Diagnosis of 11-hydroxylase deficiency is mainly based on increased 11-deoxycorticosterone concentrations in blood.

Treatment

Classic CAH patients require lifelong substitution therapy to replace missing glucocorticoids and mineralocorticoids, and consequently lower ACTH and adrenal androgen concentrations. However, in practice mostly supraphysiological dosages of glucocorticoids are needed to normalize adrenal androgen concentrations, increasing the risk on adverse effects due to the high dosages of glucocorticoids (1, 11).

Consequences of CAH

Cortisol deficiency can lead to severe complaints, such as weakness, fatigue, weight loss, and can also lead to life-threatening adrenal crises especially in situations of increased demands of cortisol (12, 13). CAH patients might also suffer from several long-term consequences due to cortisol and aldosterone deficiency, excess concentrations of androstenedione, or treatment with supraphysiological dosages of glucocorticoids, or aldosterone excess due to mineralocorticoid overtreatment. Common long-term complications are premature pubarche (14), reduced final height (15-17), impaired gonadal function (14, 17-20), decreased bone mineral density (14, 17), and an increased risk on obesity (14, 15, 17, 21), and cardiovascular morbidity (14, 17, 21).

Impairment of gonadal function in CAH males

Fertility and gonadal function can be impaired due to abnormal semen quality, Sertoli cell dysfunction, low testosterone and high gonadotropin concentrations (hypergonadotropic hypogonadism), or low testosterone and low gonadotropin concentrations (hypogonadotropic hypogonadism). Low gonadotropin concentrations are a result of negative feedback on the hypothalamus-pituitary-gonadal (HPG) axis, caused by the aromatization of increased adrenal androgens to estrogens. Gonadal function can also be impaired due to the development of testicular adrenal rest tumors (TARTs). TARTs are a common complication in CAH males and can cause gonadal dysfunction and infertility by obstruction of the seminiferous tubules or due to paracrine effects of hormones produced by TART (14, 17-20). An extensive introduction on TART will be given in our review described in chapter 4 of this thesis.

Aim of the thesis

Many processes in the body are regulated by strict hormonal balance. CAH patients suffer from the consequences of increased adrenal androgens, and decreased cortisol and aldosterone concentrations. Due to the rarity of the disease, mechanisms and related consequences of CAH are still not completely understood. The aim of this thesis is therefore to gain further insights into the disturbed adrenal steroidogenesis and its consequences in patients with CAH.

The first chapters of this thesis focus on adrenal steroidogenesis and the role of steroid precursors in CAH patients. Cortisol deficiency can lead to the development of life-threatening adrenal crises, especially during severe stress moments, as the stress response is impaired. However, not all untreated CAH patients present with the typical features of a cortisol deficiency, such as neonatal hypoglycemia or conjugated jaundice. Adrenal steroid precursors are synthesized from cholesterol and closely resemble the molecular structure of cholesterol and one another. Therefore, glucocorticoids and mineralocorticoids can bind to the glucocorticoid and mineralocorticoid receptor (GR and MR). We investigated in chapter 2 whether adrenal steroid precursors 21-deoxycortisol, 17-hydroxyprogesterone, progesterone, and androstenedione are able to activate the GR and thereby may compensate for the cortisol deficiency. In chapter 3 we continued this research by studying the potency of glucocorticoid and mineralocorticoid precursors on the GR *in vitro*. Furthermore, we described a unique cohort of Indonesian CAH patients that survived without any glucocorticoid treatment. We studied their steroid profile biochemically and linked steroid concentrations to their potency to activate the GR.

In the following chapters we focus on long-term consequences in male CAH patients. In chapter 4 we present a review on a common complication that occurs in male CAH patients: TART. TARTs are benign nodules with both testicular and adrenal characteristics and probably originate from the adrenogonadal primordium. GATA transcription factors, involved in the adrenogonadal development, were recently linked to TART development in mice. We investigated in chapter 5 the expression of GATA transcription factors in human TART and compared expression patterns to Leydig cell tumors to come up with a marker that discriminates these tumor types to prevent incorrect treatment due to a misdiagnosis. Furthermore, in chapter 6 we present an unusual case of TART in a patient with Cushing's syndrome. In chapter 7 we studied gonadal function, including the presence of TART, in a multicenter European cohort of the dsd-LIFE study. The same cohort of patients was used to investigate the quality of life in male patients with CAH in chapter 8. The thesis concludes with a summary and general discussion presented in chapter 9.

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Chapter 2

Adrenal steroid metabolites accumulating in congenital adrenal hyperplasia lead to transactivation of the glucocorticoid receptor

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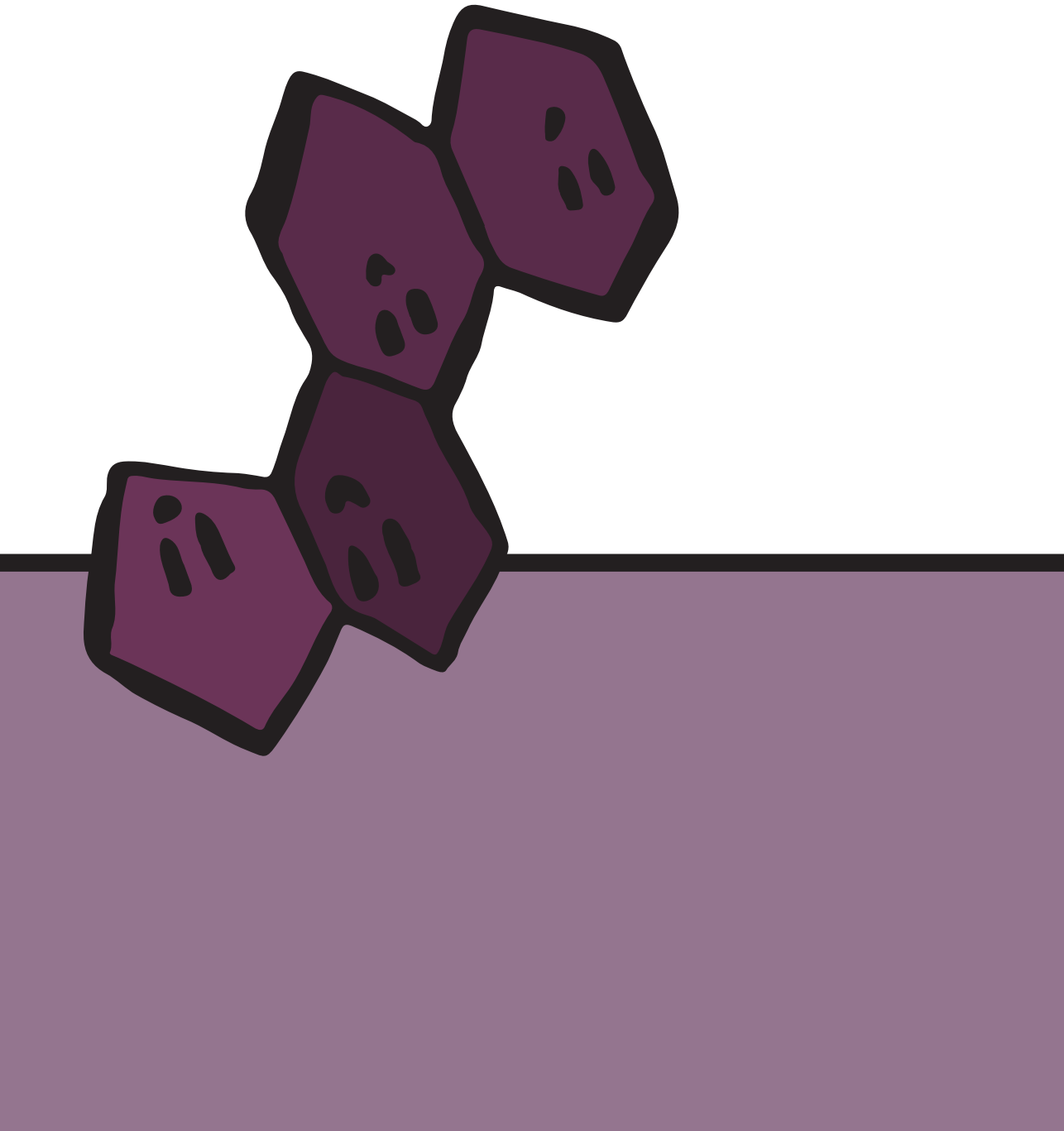
Endocrinology. 2015 Oct;156(10): 3504-3510



Abstract

Patients with congenital adrenal hyperplasia (CAH) are often clinically less severely affected by cortisol deficiency than anticipated from their enzymatic defect. We hypothesize that adrenal steroid hormone precursors that accumulate in untreated or poorly controlled CAH have glucocorticoid activity and partially compensate for cortisol deficiency. We studied the *in vitro* effects of 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, and androstenedione on the human glucocorticoid receptor (GR). Competitive binding assays were performed in HeLa cells. Nuclear translocation of the GR was studied by transfection of COS-7 cells with a GFP-tagged GR and fluorescence microscopy. Transactivation assays were performed in COS-7 cells and in HEK293 cells after cotransfection with GR and luciferase reporter vectors using a dual luciferase assay. 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol are able to bind to the GR with binding affinities of 24-43% compared with cortisol. Androstenedione has a low binding affinity. Incubation with 21-deoxycortisol led to complete nuclear translocation of the GR, whereas treatment with 17-hydroxyprogesterone or progesterone resulted in partial nuclear translocation. 21-deoxycortisol transactivated the GR with an EC_{50} approximately 6 times the EC_{50} of cortisol. 17-hydroxyprogesterone and progesterone transactivated the GR with EC_{50} values of more than 100 times the EC_{50} of cortisol. No GR transactivation was detected after incubation with androstenedione. 21-deoxycortisol, 17-hydroxyprogesterone, and progesterone are able to bind, translocate, and transactivate the GR *in vitro* and thus may have glucocorticoid activity. 21-deoxycortisol might have a clinically relevant agonistic effect on the GR and could potentially partially compensate the cortisol deficiency in CAH patients.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by a deficiency of one of the enzymes involved in adrenal steroid synthesis. Ninety-five percent of cases are caused by 21-hydroxylase deficiency. This results in impaired synthesis of cortisol. In 75% of cases patients also suffer from clinically relevant aldosterone deficiency (1). Due to the reduced negative feedback to the hypothalamus and pituitary gland, cortisol deficiency leads to increased pituitary secretion of adrenocorticotropic hormone (ACTH). Consequently, the steroid hormone precursors prior to the enzymatic block accumulate and are shunted into the androgen-synthesis pathway. The presence of increased concentrations of adrenal steroid hormone precursors is a hallmark feature of patients with CAH and these precursors can be used as diagnostic markers (2). CAH caused by 21-hydroxylase deficiency represents a spectrum of disease depending on the severity of the enzymatic defect. The most severe, classic CAH, is subdivided in a salt-wasting form and a simple virilizing form without aldosterone deficiency. Non-classic CAH is less severe with generally only mild symptoms of hyperandrogenism (1). The treatment of classic CAH consists of lifelong replacement of glucocorticoids and if necessary also of mineralocorticoids. The goal of treatment is to prevent adrenal and salt-wasting crises and to suppress abnormal secretion of adrenal androgens by suppression of the pituitary gland. Treatment of non-classic CAH is only indicated when there are severe symptoms of androgen excess and/or glucocorticoid deficiency (2).

Patients with CAH are clinically often less severely affected by cortisol deficiency than anticipated from their enzymatic defect. For example, patients with salt-wasting CAH have a severe cortisol and aldosterone deficiency due to a residual enzymatic activity of less than 1% (1). They develop salt-wasting crises neonatally, but only a minority present with hypoglycemia or conjugated jaundice, a classic symptom of infantile glucocorticoid deficiency (3). Patients with simple virilizing CAH have a residual enzymatic activity of 1-2% and a suboptimal increase of cortisol after stimulation with ACTH. In areas where neonatal screening is not implemented, male patients with simple virilizing CAH often present with signs of androgen excess in (early) childhood, without a history of Addisonian crises during illness or surgery prior to their diagnosis (1). Furthermore, many adult patients with classic CAH who are lost to endocrine follow-up and are not adherent to treatment with glucocorticoids do not develop adrenal crises for long periods of time (4). Finally, between 30 and 40% of patients with non-classic CAH diagnosed in adolescence or adulthood show a suboptimal cortisol response to ACTH stimulation, suggesting a potential risk of adrenal insufficiency during illness or surgery (5-7). However, these patients usually do not report a history

of signs or symptoms consistent with adrenal insufficiency during surgery or illness (5, 7).

These observations could hypothetically be explained by the presence of adrenal steroid hormone precursors that accumulate in patients with untreated and poorly controlled CAH. These precursors, such as 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, and androstenedione (2), have structural similarities to cortisol (8). We hypothesize that they may have clinically significant glucocorticoid activity and partially substitute for cortisol. To analyze their glucocorticoid properties, we studied their effects on binding, nuclear translocation, and transactivation of the human glucocorticoid receptor (GR).

Materials and Methods

In vitro receptor binding assays

Competitive binding assays for the GR were performed as described previously (9). HeLa cells were cultured in DMEM high glucose (4.5 g/L) with L-glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all PAA Laboratories, GmbH). Whole cells ($0.2\text{-}1.0*10^6$) were incubated in serum and phenol-red-free RPMI medium (final volume, 150 µL) for 1.5-2 hours at 37°C in a series of 0.5 mL microcentrifuge tubes containing 30 nM ^{3}H-cortisol (PerkinElmer, Inc.) and an increasing amount of unlabeled competitor: cortisol, 17-hydroxyprogesterone, 21-deoxycortisol, androstenedione (all Steraloids), or progesterone (Sigma-Aldrich). Non-specific binding was assessed by means of 500-fold excess of dexamethasone (Steraloids). Radioactivity was counted in a *J3* counter. Specific binding was expressed as the percentage of specific binding over binding of radioligand only, corrected for non-specific binding.

In vitro nuclear translocation assays

For the intracellular localization assays, GR DNA (pRShGRα) was PCR amplified using primers with BamHI and EcoRV restriction sites (Sigma-Aldrich). It was cloned into a pcDNA6-V5/HisB-EGFP vector (Invitrogen Corp.). The correct insertion of the GR construct as well as the integrity of the cDNA was checked by direct DNA sequencing.

The assays were carried out in COS-7 cells, which is a cell line that does not contain endogenous GR. The COS-7 cells were cultured in DMEM high glucose (4.5 g/L) with L-Glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. COS-7 cells ($n=1.5*10^5$) were grown in six-well plates on glass

coverslips. After 24 hours the cells were transiently transfected with 2 µg of GFP-GR using the TransIT-LT1 DNA transfection reagent (Mirus Bio). Forty-eight hours after transfection the cells were treated for 60 minutes with one of the steroids (cortisol, 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, androstenedione) in a concentration of 10^{-6}M. Afterward the cells were fixated on coverslips in 100% methanol at -20°C for 15 minutes and mounted with Vectashield with DAPI (Vector Laboratories) on microscope slides. The cells were studied under the Zeiss Apotome Fluorescence microscope with Zeiss AxioVision imaging software (version 4.7.2) at a magnification of 200X for the localization of the GR. Representative images were taken. The experiment was performed in duplicate.

In vitro transactivation assays

For the transactivation assays, the GR DNA was cloned into a pcDNA6-V5/HisB vector (Invitrogen Corp.) using the same restriction enzymes as described above. The luciferase reporter vectors used for the transactivation assays were MMTV-luc and pRL-TK (Promega). MMTV-luc is a firefly luciferase reporter construct. Transcription is controlled by glucocorticoid response elements immediately upstream from the luciferase sequence. pRL-TK contains a renilla luciferase. It serves as an internal standard to normalize firefly luciferase light emission measurements with regard to transfection efficiency and the number of cells in each well.

The COS-7 cells were cultured in DMEM high glucose (4.5 g/L) with L-glutamine supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and seeded in 24-well plates in a density of $2*10^4$ cells per well. Twenty-four hours after seeding, the COS-7 cells were transiently cotransfected using the TransIT-LT1 DNA transfection reagent with 0.2 µg pcDNA6- V5/HisB-GR, 0.3 µg MMTV-luc, and 0.01 µg pRL-TK per well. Two days after transfection the cells were treated with one of the steroids (cortisol, 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, androstenedione). Steroid solutions in ethanol were made in concentrations of 200X the final desired concentration (10^{-9} to 10^{-4}M) and diluted 1:200 with DMEM prior to adding them to the transfected cells. Twenty-four hours after adding the steroid, firefly and renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega) on a Fluoroskan FL luminometer (Thermo Scientific). Firefly luciferase/renilla luciferase ratios were calculated to normalize for transfection efficiency. Each experiment was performed in triplicate.

To ensure that the COS-7 cell line did not contain relevant amounts of endogenous steroid receptors, the transactivation activities of the different steroids were also measured in COS-7 cells that had not been transfected with steroid receptor expression vectors. Likewise, the system was tested for the presence of endogenous

steroids by measuring the transactivation activity after transfection with the GR vectors but without addition of steroids. In neither approach relevant transactivation was measured.

The transactivation assay was repeated in HEK293 cells using the methods described above. These experiments were performed in duplicate.

Statistical analysis

Analyses were performed using GraphPad Prism software version 5.0 for Windows. Steroid concentrations were expressed on a log scale and dose-response curves were calculated using nonlinear regression. For the receptor binding assays, the concentration of unlabeled steroid that reduces binding of the radioligand by half (IC_{50}) was determined. The relative binding affinity of the study steroids compared with cortisol was calculated by $IC_{50:cortisol}/IC_{50:test\ steroid} * 100\%$. For the transactivation assays, the estimated concentration for 50% transactivation (EC_{50}) was determined. For calculation of the relative functional sensitivity of the GR to the different steroids, the transactivation potential of cortisol was set at 100%. The sensitivity of the GR to the other test compounds was calculated as $EC_{50:cortisol}/EC_{50:test}$ steroid $* 100\%$.

Results

Receptor-binding assay

The receptor-binding curves for the studied steroids are shown in Fig. 2.1. The IC_{50} and the relative binding affinity of cortisol, 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, and androstenedione were calculated (Table 2.1). The results demonstrate that 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol bind to the GR with relative binding affinities of 27, 43, and 24%, respectively. The binding affinity for androstenedione is dramatically less (0.4%) than that of cortisol.

Nuclear translocation assay

Incubation with cortisol in a concentration of 10^{-6}M resulted in complete nuclear translocation of the GR within 60 minutes (Fig. 2.2B). Adding 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol to the transfected cells in a concentration of 10^{-6}M resulted in respectively almost complete, partial, and complete transport to the nucleus (Fig. 2.2C-E). After treatment of the cells with androstenedione, the location of the GR was still predominantly cytoplasmic (Fig. 2.2F).

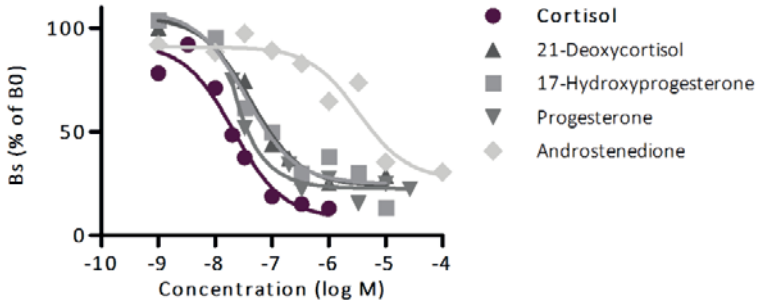


Fig. 2.1: Binding assay. Competition of various steroids for binding of ^{3}H cortisol to the glucocorticoid receptor in HeLa cells. Binding data are expressed as the percentage of specific binding (Bs) remaining after adding increasing amounts of competitor.

Table 2.1: Binding and transactivation capacity of cortisol, 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, and androstenedione to the glucocorticoid receptor.

Adrenal Steroid Metabolite	Binding Assay		Transactivation Assay in COS-7 cells		Transactivation Assay in HEK293 cells	
	IC_{50} (95% CI)	Relative binding affinity	EC_{50} (95% CI)	Relative sensi-tivity	EC_{50} (95% CI)	Relative sensi-tivity
cortisol	$2.2*10^{-8}$ ($1.3*10^{-8}$ - $3.8*10^{-8}$)	100%	$1.7*10^{-8}$ ($1.0*10^{-8}$ - $2.8*10^{-8}$)	100%	$3.5*10^{-9}$ ($1.8*10^{-9}$ - $6.9*10^{-9}$)	100%
17-hydroxy-progesterone	$8.2*10^{-8}$ ($3.8*10^{-8}$ - $1.6*10^{-7}$)	27%	$2.2*10^{-6}$ ($1.5*10^{-6}$ - $3.2*10^{-6}$)	0.8%	$1.2*10^{-6}$ ($7.6*10^{-7}$ - $1.9*10^{-6}$)	0.3%
progesterone	$5.1*10^{-8}$ ($3.1*10^{-8}$ - $7.8*10^{-8}$)	43%	$3.0*10^{-6}$ ($1.5*10^{-6}$ - $5.9*10^{-6}$)	0.6%	$2.3*10^{-6}$ ($1.2*10^{-6}$ - $4.8*10^{-6}$)	0.2%
21-deoxy-cortisol	$9.1*10^{-8}$ ($6.2*10^{-8}$ - $1.3*10^{-7}$)	24%	$1.0*10^{-7}$ ($5.2*10^{-8}$ - $2.0*10^{-7}$)	17%	$4.1*10^{-8}$ ($2.1*10^{-8}$ - $8.2*10^{-8}$)	8.5%
androstene-dione	$5.9*10^{-6}$ ($2.0*10^{-6}$ - $2.2*10^{-5}$)	0.4%	-	-	-	-

IC_{50}: estimated concentration that reduces binding of the radioligand by 50%, in mol/L. Relative binding affinity: the binding affinity of cortisol is set at 100%. The binding of the other steroids to the GR is calculated as $IC_{50:cortisol}$/ $IC_{50:test\ steroid}$ * 100%. EC_{50}: Estimated concentration for 50% transactivation, in mol/L. Relative sensitivity: the transactivation potential of cortisol is set at 100%. The sensitivity of the GR to the other test compounds is calculated as $EC_{50:cortisol}/EC_{50:test\ steroid}$ * 100%.

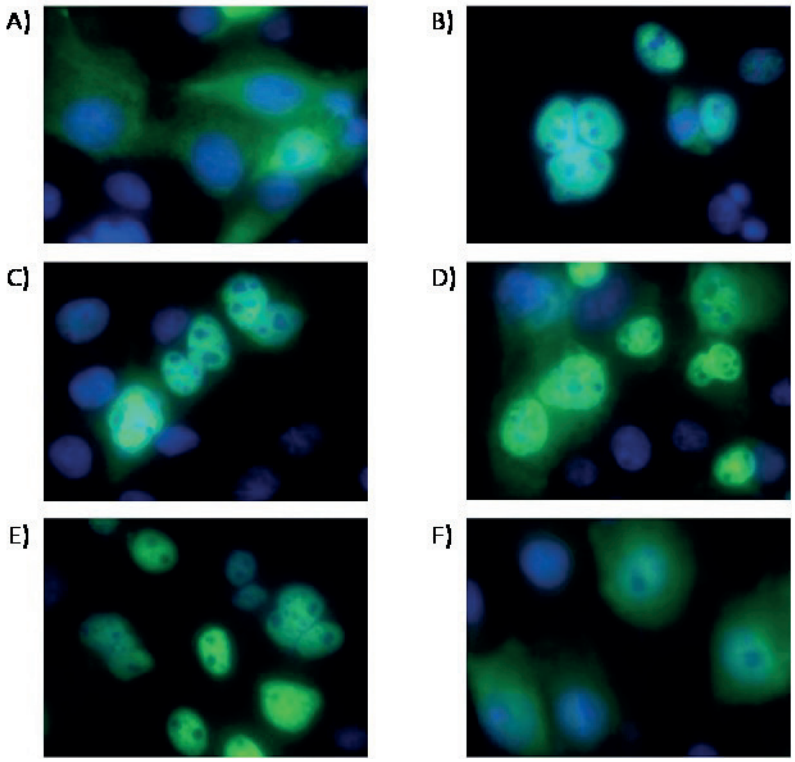


Fig. 2.2: Localization of the GR in COS-7 cells transfected with the GR, without steroids (A) and after incubation with various steroids in a concentration of 10^{-6}M (B–F). The nucleus is stained blue and the GR is tagged with a green fluorescent protein. **B**, cortisol. **C**, 17-hydroxyprogesterone. **D**, progesterone. **E**, 21-deoxycortisol. **F**, androstenedione.

GR transactivation assay in COS-7 cells

Cortisol activated the GR with an EC_{50} of $1.7*10^{-8}$M (Fig. 2.3; Table 2.1). Exposure of the GR to increasing concentrations of 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol resulted in increasing GR transactivation, up to a maximum. A dose-response curve was fitted (Fig. 2.3) and the EC_{50} values for these steroids were calculated. The EC_{50} value was $2.2*10^{-6}$M for 17-hydroxyprogesterone, $3.0*10^{-6}$M for progesterone, and $1.0*10^{-7}$M for 21-deoxycortisol (Table 2.1). Androstenedione did not transactivate the GR at the concentrations tested (Fig. 2.3).

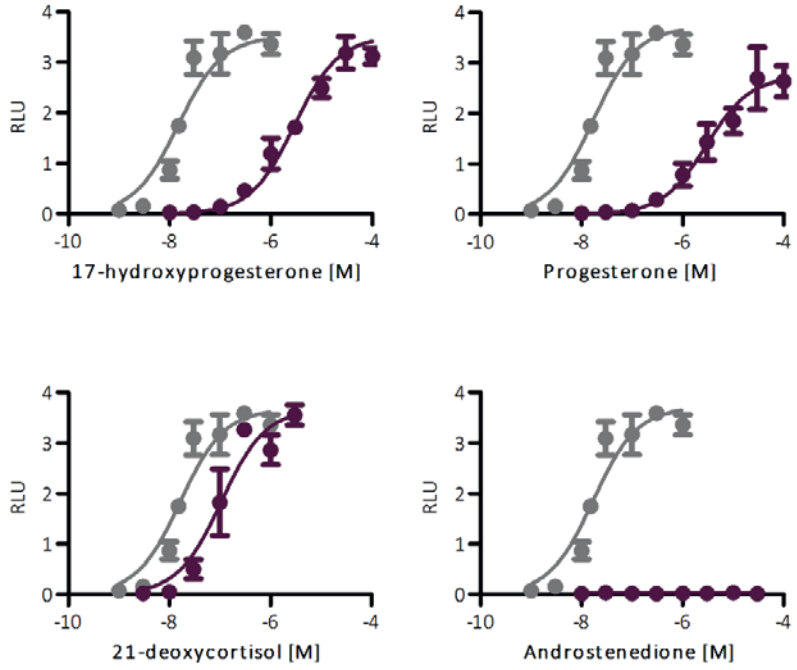


Fig. 2.3: Transactivation of the glucocorticoid receptor (GR) in COS-7 cells by 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, and androstenedione in comparison with the transactivation of the GR by cortisol (indicated in gray).

GR transactivation assay in HEK293 cells

The results for the transactivation assay in HEK293 cells are shown in Fig. 2.4 and Table 2.1. In these experiments, the EC_{50} for cortisol was $3.5*10^{-9}$M. 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol activated the GR with EC_{50} values of $1.2*10^{-6}$M, $2.3*10^{-6}$M, and $4.1*10^{-8}$M, respectively.

Discussion

We describe here the binding of 17-hydroxyprogesterone, progesterone, 21-deoxycortisol, and androstenedione to the GR and their effects on the nuclear translocation and transactivation of the GR, in comparison with the effects of cortisol.

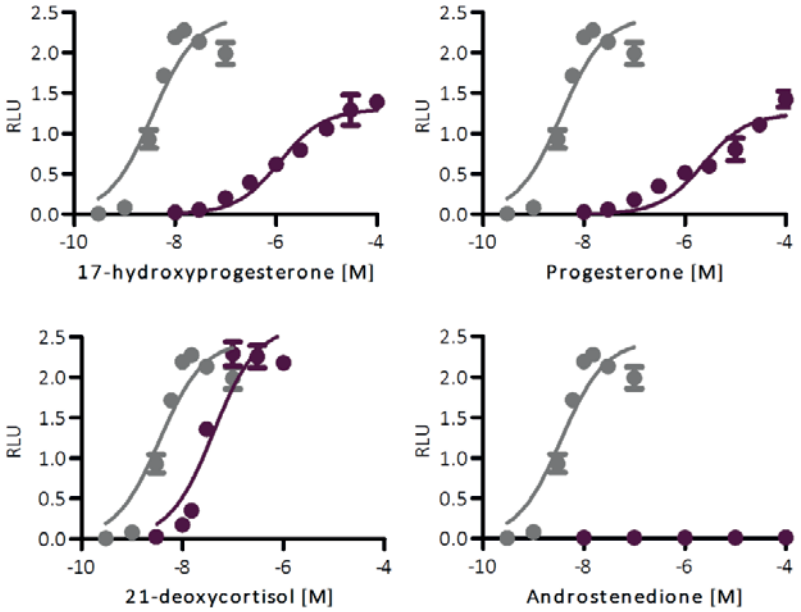


Fig. 2.4: Transactivation of the human glucocorticoid receptor (GR) in HEK293 cells by 17-hydroxyprogesterone, 21-deoxycortisol and androstenedione in comparison with the transactivation of the GR by cortisol (indicated in gray).

We found that 21-deoxycortisol and to a lesser extent 17-hydroxyprogesterone and progesterone can transactivate the GR. This is consistent with the receptor-binding and nuclear translocation of the GR we found after incubation with these steroids. It has previously been described that 17-hydroxyprogesterone and progesterone bind to the GR (10-12), but to the best of our knowledge this has not been shown for 21-deoxycortisol previously. These agonistic properties on the GR might explain the clinical observation that CAH patients are often less severely affected by cortisol deficiency than anticipated from their enzymatic defect.

Steroid hormone cross reactivity to receptors is a well-known phenomenon. For example, cortisol is known to have an agonistic effect on the mineralocorticoid receptor (13). In addition, in a previous study of our group we have demonstrated that 17-hydroxyprogesterone and progesterone can influence the transactivation of the human mineralocorticoid receptor: both have anti-mineralocorticoid effects *in vitro* (14). The occurrence of cross reactivity of these steroids at both the GR and the human

mineralocorticoid receptor can be explained by the high degree of homology at the DNA- binding domains of these receptors (15).

The EC_{50} of cortisol in our model is comparable to the EC_{50} previously described (16), demonstrating the reliability of our *in vitro* model. Under the experimental conditions 21-deoxycortisol, 17-hydroxyprogesterone, and progesterone have the capacity to transactivate the GR. With the concentrations used in our experiments we were able to reconstruct complete dose-response curves, up to the point where maximum transactivation was reached. In the two cell lines studied, a comparable profile was found: 21-deoxycortisol has the greatest transactivation capacity, 17-hydroxyprogesterone and progesterone are also able to transactivate the GR but only at much higher concentrations. Androstenedione does not transactivate the GR at the concentrations tested. Given that there was no transactivation detectable for androstenedione even in concentrations at least 100 times higher than the concentrations found in untreated CAH, we do not expect that relevant transactivation will occur with higher concentrations (17). The fact that androstenedione has no agonistic effect on the GR might be explained by the greater structural dissimilarity between androstenedione and cortisol compared with that of the other test compounds and cortisol (8).

As illustrated by the EC_{50} values more than 100 times higher than the EC_{50} of cortisol, 17-hydroxyprogesterone and progesterone are less potent agonists of the GR than cortisol. In healthy volunteers the 17-hydroxyprogesterone concentration is substantially lower than that of cortisol: reference values for serum 17-hydroxyprogesterone are 2.0-10.8 nmol/L for males and 0.45-12.7 nmol/L for females, 0900-h reference values for serum cortisol are 190-550 nmol/L. In this situation cross-reactivity on the GR will be negligible. However, in patients with classic CAH 17-hydroxyprogesterone concentrations can increase to very high concentrations of more than 1500 nmol/L (18, 19). Based on the dose-response curve we constructed, these concentrations might be high enough to result in relevant GR transactivation.

Interestingly, the EC_{50} of 21-deoxycortisol is much closer to the EC_{50} of cortisol (approximately 6-fold in the transactivation assays in COS-7 cells, approximately 12-fold in the HEK293 cells). The serum concentrations of 21-deoxycortisol in untreated or poorly controlled patients with classic CAH can exceed 400 nmol/L (18, 19). Based on our results we hypothesize that these concentrations may lead to a clinically relevant transactivation of the GR. Less transactivation can be expected in non-classic CAH patients, given that in these patients the 21-deoxycortisol concentrations are lower and reach up to approximately 40 nmol/L (6). We suggest that high serum 21-deoxycortisol concentrations in untreated or poorly controlled CAH patients may

partially compensate for their cortisol deficiency. In contrast, overtreatment with complete suppression of adrenal precursors including 17-hydroxyprogesterone and 21-deoxycortisol might lead to an increased risk of adrenal crises and hypoglycemia. Therefore, adequate stress dosing is crucial once glucocorticoid treatment is initiated in patients with CAH.

Considering that we have studied several elements of the GR transactivation cascade with consistent results, we are confident that our results represent actual glucocorticoid properties of 21-deoxycortisol, 17-hydroxyprogesterone, and progesterone. However, given that this is an *in vitro* model, our findings must be confirmed in additional studies. We have studied the transactivation properties of several steroids and not the other mechanisms by which the glucocorticoid receptor exerts its actions: transrepression and nongenomic effects (13, 20, 21). It might also be relevant to study other steroid precursors. Despite these limitations, we consider our results promising and potentially relevant for a significant group of patients with CAH.

In conclusion, 21-deoxycortisol and to a lesser extent 17-hydroxyprogesterone and progesterone are able to transactivate the GR *in vitro* and thus may have glucocorticoid activity. 21-deoxycortisol, which can be strongly elevated in patients with untreated or poorly controlled CAH, has the strongest agonistic effect on the GR and may partially compensate for the cortisol deficiency in patients with CAH.

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Disclosure Summary: The authors have nothing to disclose.

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Chapter 3

Adrenal steroid precursors with glucocorticoid activity are able to prevent adrenal crises in untreated congenital adrenal hyperplasia patients

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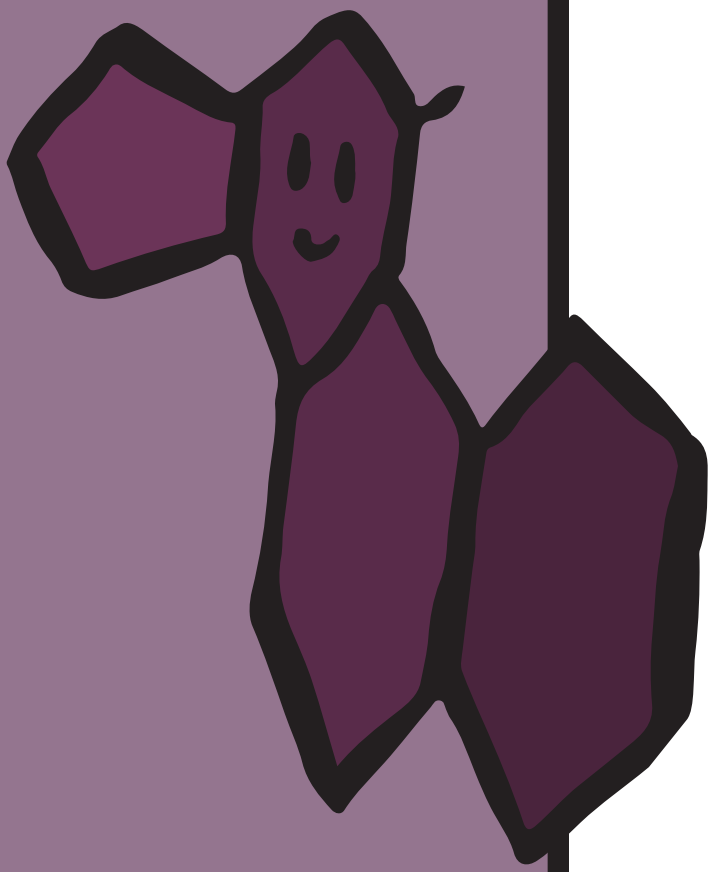
Submitted



Abstract

Objective: Cortisol deficiency can cause adrenal crises, which can be life-threatening. Cortisol production is impaired in patients with congenital adrenal hyperplasia (CAH). Here, we describe a unique population of severely affected untreated CAH patients, with proven cortisol deficiency but without clinical signs of cortisol deficiency even in severe stress-situations. The lack of clinical signs of cortisol deficiency in untreated CAH patients might be explained by the glucocorticoid activity of increased concentrations of adrenal steroid precursors.

Design: Translational research.

Methods: Adrenal steroid precursor concentrations before and 60 minutes after adrenocorticotropic hormone (ACTH) administration of 22 untreated Indonesian CAH patients (3-46 years) with proven cortisol deficiency (<500 nmol/L post-ACTH) measured by liquid chromatography tandem-mass spectrometry (LC-MS/MS) were compared to 6 control patients (Mann-Whitney U test). Glucocorticoid activity was determined by dual-luciferase assays in human embryonic kidney cells transfected with the glucocorticoid receptor and exposed to increasing amounts of adrenal steroid precursors for 24 hours.

Results: Blood concentrations of the steroid precursors 11-deoxycortisol (457 nmol/L, p=0.003), 11-deoxycorticosterone (55 nmol/L, p=0.003), 17-hydroxyprogesterone (610 nmol/L, p<0.001), progesterone (29 nmol/L, p<0.001), and 21-deoxycortisol (73 nmol/L) were strongly elevated compared to controls. The GR was activated with comparable potency to cortisol by corticosterone and 21-deoxycortisol or with 4-100x lower potency by 11-hydroxyprogesterone, 11-deoxycortisol, aldosterone, 11-deoxycorticosterone, progesterone, and 17-hydroxyprogesterone.

Conclusions: We identified strongly elevated adrenal steroid precursor concentrations in blood from untreated CAH patients and demonstrated glucocorticoid activity of these adrenal precursors *in vitro* – implicating these precursors are able to compensate for cortisol deficiency in untreated CAH patients, thereby protecting against life-threatening adrenal crises.

Introduction

Adrenal crisis due to cortisol deficiency, also known as Addisonian crisis, is a life-threatening condition when not properly treated with glucocorticoids. It is often described by its main symptoms: vomiting, diarrhea, and abdominal pain. Whereas the main cause of an adrenal crisis is cortisol deficiency, additional aldosterone deficiency can lead to the development of a salt-wasting crisis, which is treated by supplementation of mineralocorticoids and sodium chloride.

Cortisol deficiency is one of the main findings in patients with congenital adrenal hyperplasia (CAH). CAH is an autosomal recessive disorder with impaired steroidogenesis in the adrenal cortex. Adrenal steroidogenesis involves the synthesis of cortisol, aldosterone, and androgens from the common precursor cholesterol via several enzymatic steps. More than 90% of CAH cases are caused by a mutation in the *CYP21A2* gene resulting in 21-hydroxylase deficiency (21OHD). In 21OHD, conversion of the steroid precursors 17-hydroxyprogesterone and progesterone to 11-deoxycortisol and 11-deoxycorticosterone, respectively is impaired (Fig. 3.1). Consequently, cortisol production is inadequate, leading to elevated adrenocorticotropic hormone (ACTH) concentrations due to a lack of negative feedback to the hypothalamus and pituitary gland. Increased ACTH concentrations will stimulate the adrenal gland, eventually causing hyperplasia. Furthermore, adrenal steroid precursors before the enzymatic defect accumulate and are shunted into the androgen synthesis pathway (1). Increased concentrations of adrenal steroid precursors 17-hydroxyprogesterone and 21-deoxycortisol are currently used as diagnostic markers for CAH (1-3). The severity of the disease depends on the residual enzymatic activity with a strong phenotype-genotype relationship: Mutations that are classified as group null (0) or A are usually associated with <1% residual enzyme activity with additional aldosterone deficiency (classic salt-wasting type), group B with 1-5% residual enzyme activity without aldosterone deficiency (classic simple virilizing type), and group C with a residual enzymatic activity of 20-50% and a less severe phenotype with usually normal cortisol and aldosterone levels (non-classic type) (4).

Approximately 5% of CAH cases are caused by mutations in the *CYP11B1* gene resulting in 11-hydroxylase deficiency (11OHD). 11OHD results in accumulation of the precursors 11-deoxycorticosterone and 11-deoxycortisol (Fig. 3.1). As in 21OHD, cortisol production is deficient and adrenal androgen concentrations are increased. However, in contrast to 21OHD, signs of mineralocorticoid excess occur with hypertension and hypokalemia due to the mineralocorticoid potency of 11-deoxycorticosterone (5).

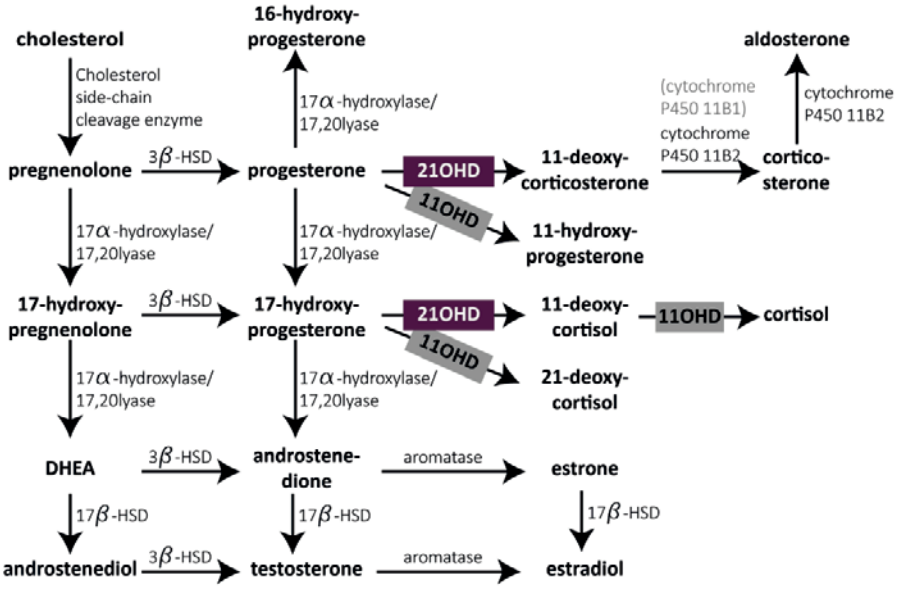


Fig. 3.1: Adrenal steroidogenesis. Cholesterol is converted to aldosterone, cortisol, and androgens in the adrenal cortex via several enzymatic steps. Abbreviations: 11OHD, 11-hydroxylase deficiency; 21OHD, 21-hydroxylase deficiency; HSD, hydroxysteroid dehydrogenase.

Patients with cortisol deficiency are generally treated with glucocorticoids to substitute for cortisol deficiency in order to prevent life-threatening adrenal crises and to suppress the elevated androgens. In our clinical experience however, there are cases of untreated or noncompliant severely affected CAH patients known to have less clinical signs of cortisol deficiency than expected based on their enzyme deficiency and compared to patients with other forms of adrenal insufficiency. For example, only a minority of neonates with the most severe form of 21OHD present with typical features of cortisol deficiency (hypoglycemia or conjugated jaundice) (6). We have previously described a patient with salt-wasting CAH, who was only treated with sodium chloride for the first two years of life, without apparent complications from cortisol deficiency (7). Moreover, in countries without neonatal screening programs for CAH, CAH patients from group B (simple virilizing) usually only present with signs of androgen excess during childhood, without reporting a history of adrenal crises.

The aim of our study was to determine if this lack of signs of cortisol deficiency might be explained by the glucocorticoid activity of increased concentrations of adrenal steroid precursors that accumulate before the enzymatic defect. Here, we describe the clinical phenotype and the biochemical profile of adrenal steroid precursors in a unique cohort of untreated 21OHD and 11OHD CAH patients, before and after ACTH

administration using liquid chromatography tandem-mass spectrometry (LC-MS/MS). The glucocorticoid activity of these precursor steroids was assessed by *in vitro* glucocorticoid receptor (GR) transactivation studies.

Subjects and Methods

Study design

Within this translational study we had the objective to describe the phenotype and steroid profile of a cohort of untreated Indonesian CAH patients. No sample size calculation was performed in this descriptive study. We added 6 control patients and 2 untreated CAH patients from the Netherlands in comparative biochemical analyses. Our inclusion criteria were untreated patients with a severe type of genetically confirmed CAH in which an ACTH stimulation test was performed. To understand the potential of different steroid hormones, we also performed cell culture experiments. We determined the potency to activate the GR for each steroid, in which each concentration was measured in triplicate. No rules for stopping data collections were defined. We reported all data and did not find outliers in our data. Main outcomes in our study were defined as the number of stress-full events, biochemical evaluation of the ACTH stimulations tests by LC-MS/MS and activation of GR.

Patients

Untreated CAH patients (n=22) were recruited from the local CAH database and included as a part of a cohort study on hormonal analysis and compliance in the Center for Biomedical Research, Faculty of Medicine Diponegoro University, Semarang, Indonesia (in compliance with relevant laws, institutional guidelines and the declaration of Helsinki). The study was approved by the local ethical committee of the Diponegoro University. Oral and written informed consent was obtained after full explanation of the purpose and nature of all procedures. Data were collected on the type of CAH, karyotype, gender, mutation analysis, signs of salt-wasting, and episodes of severe stress in their medical history (critical illness, trauma, surgery) by the local pediatric endocrinologist (AU). For all 21OHD patients, the severity of CAH was classified based on their mutation analysis into genotype groups 0, A, B, or C (4).

In addition, the biochemical results of patients in whom an ACTH test was performed at Radboud university medical center in Nijmegen, the Netherlands in 2017 were included (n=8). Oral informed consent was obtained by the treating physician as additional measurements were performed on coded stored blood samples in accordance with the

Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/codes-conduct). Two patients had a CAH diagnosis, the other 6 were non-CAH patients, falsely suspected of an adrenal disorder, and were used as control group. Three of these patients were females with polycystic ovarian syndrome. One male patient presented with an abnormal result in the neonatal screening program for CAH, which turned out to be false positive, and the 2 other patients were 46XY neonates with ambiguous genitalia. In one of them, no final diagnosis could be made. In the other patient, a mutation with unknown pathogenicity was found in the NR5A1 gene. Additionally, 1 healthy adult female underwent an ACTH stimulation test in the Center for Biomedical Research, Faculty of Medicine Diponegoro University, Semarang, Indonesia to serve as a control patient.

Biochemical analysis

The standard ACTH stimulation test was performed in all patients to determine adrenal functioning by measuring steroid concentrations before and after ACTH administration. Synacthen (0.25 mg; Sigma Tau BV) was injected intravenously, with blood draws for steroid analysis before and 60 minutes after the injection. In the Indonesian patients all tests were performed before 9.00 am. Dutch patients had their ACTH stimulation test during their clinical appointment, which mostly took place in the morning. None of the patients did receive glucocorticoids at the time of biochemical analysis. Serum before and after ACTH administration were analyzed with LC-MS/MS to determine cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol concentrations. The LC-MS/MS protocol has been provided in the Supplemental Material.

In vitro transactivation study

Cell culture

Human embryonic kidney cells (HEK293) were grown as a monolayer culture in DMEM with 4.5 g/L glucose with L-glutamine (Lonza; Leusden, the Netherlands) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific; Landsmeer, the Netherlands) and 1% antibiotics (penicillin-streptomycin 10 000 U/mL; Gibco). Cells were cultured at 37°C in a humidified 95% air / 5% CO_2 atmosphere and passaged when confluent.

In vitro dual luciferase transactivation assays

Human GR transactivation was measured using dual-luciferase transactivation assays (Promega; Leiden, the Netherlands) in which pcDNA6-V5/HisB-hGR, MMTV-luc, and pRL-TK vectors were used as described earlier (8).

HEK293 cells were seeded at 40 000 cells/well in 24-well plates. Transient transfection was performed after 24 hours, using 0.2 µg pcDNA6-V5/HisB-hGR, 0.3 µg MMTV-luc, and 0.01 µg pRL-TK per well and 1 µL TransIT-LT1 transfection reagent (Mirus; Ochten, the Netherlands) according to manufacturer's protocol. Cells were treated for 24 hours with one of the steroids (progesterone, 17-hydroxypregnenolone, pregnenolone (Sigma-Aldrich, Zwijndrecht, the Netherlands) or cortisol, aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxyprogesterone, 11β-hydroxyprogesterone, 16-hydroxyprogesterone, 21-deoxycortisol, (Steraloids, Newport, USA)) 2 days after transfection. Steroid solutions were prepared in ethanol (200X concentrated) and diluted 1:200 in culture medium prior to treatment. Thereafter, firefly and renilla luciferase activity was measured on a Fluoroskan FL luminometer (Thermo Scientific) according to manufacturer's protocol (Promega) and firefly/renilla ratios were calculated. Each concentration was measured in triplicate.

As controls, HEK293 cells were transfected with the MMTV-luc and pRL-TK vector but not the GR and treated with 10^{-4}M cortisol. Secondly, HEK293 cells were transfected with all the vectors but not treated with any steroid (medium with 0.5% ethanol). Neither of these approached resulted in transactivation activity.

Statistical analyses

GraphPad Prism software version 5.0 for Windows and SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA) were used to analyze adrenal steroid concentrations. Normality was assessed and median steroid concentrations and interquartile ranges (IQR: Q1-Q3) were calculated. 21OHD and 11OHD patients were compared to the control group and distributions were compared using the Mann-Whitney U (MWU) test. $P<0.05$ was considered significant. GraphPad Prism was also used to calculate dose-response curves using nonlinear regression. The estimated concentration that causes 50% of the maximum transactivation (EC_{50}) and its 95% confidence interval (95%CI) were also determined. Relative functional sensitivity of the GR to the different steroids was calculated as $EC_{50\ cortisol}$ / $EC_{50\ test\ steroid}$ x 100% in which cortisol was set as 100%.

Results

Clinical characteristics of untreated CAH patients

Clinical characteristics of the included CAH patients are shown in Table 3.1. Twenty-two (long-time) untreated CAH patients were identified in the Indonesian CAH database, age 3-46 years, of which 17 had 21OHD and 5 had 11OHD. Most patients (n=15) were

Table 3.1: Clinical characteristics of untreated CAH patients.

#	age	CAH type	Karyo-type	Gen-der	Mutations	Geno-type^	Treatment with GCs	History of SW	History of severe stress while untreated	cortisol before ACTH	cortisol after ACTH
I1	28	11OHD	46XX	F	c.799G>A c.799G>A	NA	never	no	genital surgery	164	163
I2	19	11OHD	46XX	F	c.799G>A c.799G>A	NA	never	no	no	218	225
I3	5	11OHD	46XX	M	c.799G>A c.799G>A	NA	never	no	no	150	155
I4	13	11OHD	46XX	M	c.799G>A c.799G>A	NA	never	no	no	179	180
I5	3	11OHD	46XX	U	c.799G>A c.799G>A	NA	never	no	no	202	202
I6	19	21OHD	46XX	F	p.Ile172Asn p.Ile172Asn	B	treated at age 8-16yr stopped >3yrs	no	no	110	125
I7	23	21OHD	46XX	F	p.Ile172Asn p.Ile172Asn	B	treated at age 13-20yr stopped >3yrs	no	no	233	238
I8	21	21OHD	46XX	F	p.Arg356Trp p.Arg356Trp	0	treated at age 10-19yr stopped >2yrs	yes	genital surgery	68	66
I9	9	21OHD	46XX	F	intron splice p.Arg356Trp	A	treated at age 5-7yr stopped >2yrs	yes	hospital admissions (SW crises)	82	73
I10	4	21OHD	46XX	F	intron splice p.Arg356Trp	A	treated at age 0-2yr stopped >2yrs	yes	hospital admissions (SW crises)	35	33
I11	13	21OHD	46XX	F	p.Arg356Trp p.Arg356Trp	0	treated in childhood stopped >4yrs	no	genital surgery	115	119
I12	46	21OHD	46XX	M	p.Trp20* p.Ile172Asn	B	never	no	2x severe gastritis typhoid fever	159	159
I13	15	21OHD	46XX	M	intron splice intron splice	A	never	no	no	89	79
I14	32	21OHD	46XX	M	intron splice intron splice	A	never	no	genital surgery	61	65
I15	13	21OHD	46XX	F	p.Arg356Trp p.Arg356Trp	0	treated at age 5yr stopped >8yrs	no	genital surgery tonsillectomy dengue typhoid fever	75	68
I16	15	21OHD	46XX	M	p.Arg356Trp deletion of exon 1-3	0	never	yes	hospital admissions for vomiting and seizures	57	58
I17	14	21OHD	46XX	M	p.Ile386del p.Arg356Trp	0	never	yes	hospital admissions for seizures	53	50
I18	14	21OHD	46XX	F	p.Pro30Leu p.Pro30Leu	B	treated at age 4-10yr stopped >4yrs	no	genital surgery	210	187

I19	10	21OHD	46XX	M	p.Gln196* p.Arg356Trp	0	never	yes	dengue hospital admission for vomiting	61	60
I20	19	21OHD	46XX	F	p.Ile172Asn deletion of exon 1-6	B	never	no	no	139	129
I21	3	21OHD	46XX	F	p.Ile172Asn p.Arg356Trp	B	never	no	no	101	64
I22	21	21OHD	46XX	F	deletion of exon 1-6 NSM[#]	NC	never	no	tonsillectomy dengue	146	149
D1	0	21OHD	46XY	M	deletion of exon 1-3 p.Ile172Asn	B	started after ACTHstimulation test		NA	30	70
D2	0	21OHD	46XY	M	deletion of exon 1-7 p.Pro30Leu	B	started after ACTHstimulation test		NA	30	90

^Genotype classification is based on mutation analysis. Mutations in genotype group 0 and A have usually <1% residual enzyme activity, genotype B has 1-5% residual enzyme activity, while genotype C has a residual enzyme activity of 20-50% (4). We classified the p.Pro30Leu mutation in genotype group B. Cortisol concentrations are reported in nmol/L. *This patient also had a cluster of mutations in the CYP21A2 promoter region in one allele, possibly resulting in a more severe phenotype. [#]This patient had very high 17-hydroxyprogesterone levels (>400 nmol/L), and although no second mutations has been found it is very likely that this patient belongs to the same genotype group (B) as her sister (#I20). Abbreviations: 11OHD, 11-hydroxylase deficiency; 21OHD, 21-hydroxylase deficiency; dengue, dengue hemorrhagic fever; F, female; GCs, glucocorticoids; M, male; NA, not applicable; NC, not classifiable; NSM, no second mutation reported; SW, salt-wasting; U, undefined; yr, year; yrs, years.

never treated with glucocorticoids, and 7 patients were treated in the past but had been off treatment for at least 2 years at the time of biochemical analysis and were therefore considered as untreated. Additionally, we included the biochemical results of 2 Dutch male CAH neonates, who were identified from the national CAH neonatal screening program and started glucocorticoid treatment only after the biochemical analysis of the ACTH stimulation test. All patients were diagnosed with a severe type of CAH based on mutation analysis. Episodes of severe stress in their medical history has been reported in 13/22 patients. In none of these episodes patients were treated with stress dosages of glucocorticoids. In the 11OHD group, 1 patient underwent genital surgery. In the 21OHD group, 5 patients underwent genital surgery (genotype group 0 (n=3), A (n=1), B (n=1)) and 2 patients underwent tonsillectomy (genotype group 0 (n=1), unclassifiable (n=1)). There were 5 reports of dengue hemorrhagic fever (genotype group 0 (n=2), unclassifiable (n=1)) or typhoid fever (genotype group 0 (n=1), B (n=1). Also, 5 patients reported hospital admissions because of salt-wasting crisis (genotype group A (n=2)) or because of episodes of vomiting and/or seizures (genotype group 0 (n=3)).

Biochemical evaluation of serum adrenal steroid precursors

In control patients, the cortisol concentration increased adequately after ACTH administration (>500 nmol/L (9-11)) and all other measured steroids remained low with a 2-5 fold increase. In contrast, in our study population cortisol concentrations remained low after ACTH administration with a median concentration of 73 (IQR 64-129) nmol/L in the 21OHD patients and 180 (IQR 159-214) nmol/L in the 11OHD patients, confirming severe cortisol deficiency in our cohort (Table 3.1, Fig. 3.2). Adrenal precursor steroid concentrations were also measured in serum from CAH patients before and after ACTH administration (Fig. 3.2). As we did not find differences in steroid concentrations before and after ACTH administration, we further only report on the steroid concentrations in serum after ACTH administration. Corticosterone concentrations were significantly lower in untreated CAH patients (median 21OHD: 7.1 IQR 5.9-8.2) nmol/L, MWU p<0.001; median 11OHD: 4.3 (IQR 3.7-8.4) nmol/L, MWU

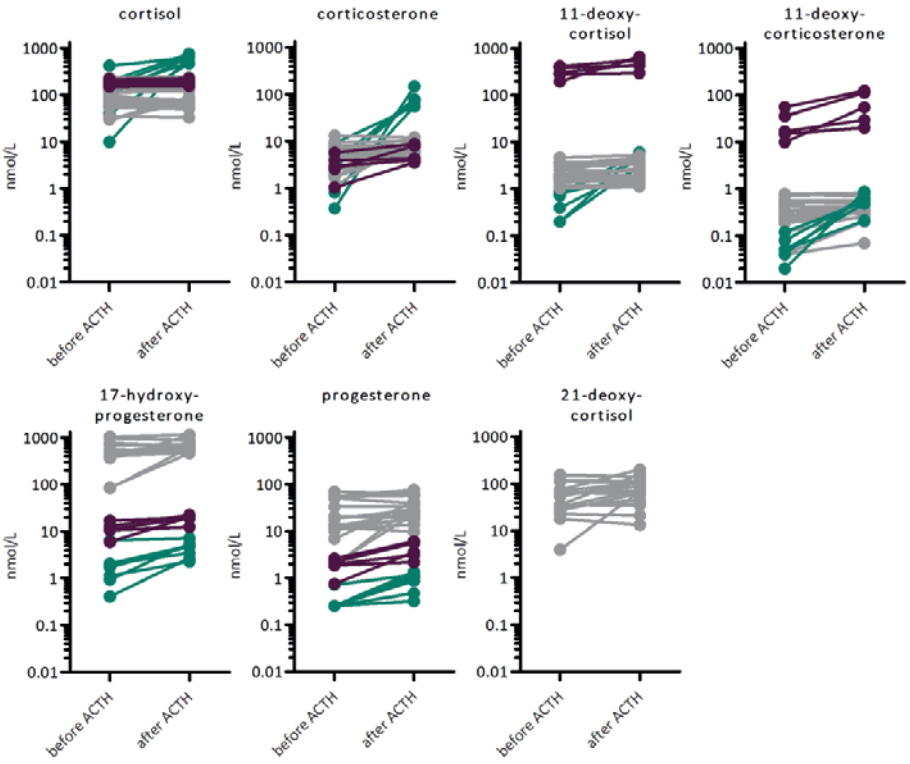


Fig. 3.2: Steroid concentrations in untreated CAH patients. Blood adrenal steroid concentrations were determined before and 60 minutes after ACTH administration. Gray indicates 21-hydroxylase deficient patients, purple indicates 11-hydroxylase deficient patients, and green indicates control.

(p=0.003) compared to controls (median 72 (IQR 55-78) nmol/L). Blood 11-deoxycortisol and 11-deoxycorticosterone concentrations were significantly elevated in 11OHD patients with respectively a median concentration of 457 nmol/L (IQR 364-612, MWU p=0.003) and 55 nmol/L (IQR 25-119, MWU p=0.003) compared to control (median 3.2 (IQR 1.9-5.9), and 0.5 (IQR 0.2-0.8) nmol/L, respectively), but not in 21OHD patients (median 1.9 (IQR 1.5-3.8, MWU p=0.083) and 0.4 (IQR 0.3-0.6, MWU p=0.395) nmol/L, respectively). Blood 17-hydroxyprogesterone and progesterone concentrations were elevated in 21OHD patients (median 610 (IQR 509-762, MWU p<0.001) and 29 (IQR 20-43, MWU p<0.001) nmol/L, respectively) and 11OHD patients (median 20 (IQR 16-21, MWU p=0.003), and 3.6 (IQR 2.6-5.8, MWU p=0.003) nmol/L, respectively) compared to controls (median 4.8 (IQR 2.7-5.6) nmol/L and 1.0 (IQR 0.5-1.1) nmol/L, respectively). 21-deoxycortisol concentrations were only measurable (>1 nmol/L) in blood from untreated 21OHD patients (median 73 (IQR 46-112) nmol/L).

GR transactivation in human embryonic kidney cells

To determine the potency of steroid precursors for GR transactivation, we performed dual-luciferase assays, which allows for the quantification of GR-induced gene expression (Table 3.2, Fig. 3.3). We found an EC_{50} of 11 nM (95%CI: 5.9-20) for cortisol, which is used as reference steroid. Similar potencies were found for corticosterone (EC_{50} of 17 nM, 95%CI: 8.9-32) and 21-deoxycortisol (EC_{50} of 22 nM, 95%CI: 12-42) as confidence intervals overlap. 11-hydroxyprogesterone, 11-deoxycortisol, and aldosterone exhibited somewhat higher EC_{50} values: 47, 71, and 111 nM, respectively. Exposure to 11-deoxycorticosterone (EC_{50} of 725 nM), progesterone (EC_{50} of 1147 nM), and 17-hydroxyprogesterone (EC_{50} of 1668 nM) also resulted in GR transactivation, although these EC_{50} values were at least 65 times higher than that of cortisol. GR transactivation was only observed at a very high concentration (100 000 nM) of 16-hydroxyprogesterone, while no GR transactivation was found for pregnenolone and 17-hydroxypregnenolone.

Discussion

Here, we describe a unique group of untreated CAH patients with biochemically confirmed severe cortisol deficiency. None of these patients had clinical signs of cortisol deficiency and more than half of these patients report a history of severe stress-situations, such as surgery or severe infectious diseases, and recovered without glucocorticoid stress dosing. We show here that this might be explained by the glucocorticoid activity of significantly increased steroid precursor concentrations, that at least partially compensate the cortisol deficiency.

Table 3.2: (Relative) potency of steroids to activate the glucocorticoid receptor (GR).

	EC_{50} (95%CI) nM	Relative potency to GR
cortisol	**11 (5.9 - 20)**	**100%**
corticosterone	17 (8.9 - 32)	64%
21-deoxycortisol	22 (12 - 42)	49%
11-hydroxyprogesterone	47 (30 - 73)	23%
11-deoxycortisol	71 (56 - 92)	15%
aldosterone	111 (72 - 171)	9.8%
11-deoxycorticosterone	725 (422 - 1246)	1.5%
progesterone	1147 (664 - 1981)	0.95%
17-hydroxyprogesterone	1668 (1300 - 2140)	0.65%
16-hydroxyprogesterone	NC*	
pregnenolone	NA	
17OHpregnenolone	NA	

Data of the transactivation assays in human embryonic kidney cells were used to calculate dose-response curves using nonlinear regression. EC_{50} values could be calculated when a complete dose-response curves was available, including a concentration in which maximum transactivation was reached (plateau phase). * some transactivation at 100 000 nM, but no maximum transactivation was reached with the concentrations tested. Abbreviations: 95%CI, 95% confidence interval; EC_{50}, estimated concentration that causes 50% of the maximum transactivation; GR, glucocorticoid receptor; NA, not applicable; NC, not calculable.

To the best of our knowledge we are the first to report on measurement of multiple adrenal steroid precursors in an unique cohort of untreated CAH patients. As expected from their enzymatic defect, in untreated 21OHD patients 17-hydroxyprogesterone (127x higher), progesterone (29x higher), and 21-deoxycortisol (only measurable in 21OHD) concentrations were significantly increased compared to control. Remarkably, this corresponds to the measured concentrations of adrenal steroid precursors in blood of treated 21OHD patients (12). Furthermore, in untreated 11OHD patients, we found 11-deoxycortisol (457x higher) and 11-deoxycorticosterone (55x higher) to be the most important accumulating steroid precursors. We hypothesized that these strongly elevated adrenal precursor concentrations may have a stimulating effect on the GR.

Steroid hormone action is initiated by binding of the hormone to its receptor. The hormone-receptor complex subsequently will translocate to the nucleus were it binds to hormone response elements in the regulatory region of target gene promoters, initiating transactivation (13). Several studies have been performed evaluating the binding of steroids to the GR and mineralocorticoid receptor (MR) and/or the nuclear translocation of the receptor-complex, but only a few studies report on GR transactivation (8, 14-18), mainly focusing on aldosterone and cortisol, showing that cortisol has higher potency for GR transactivation than aldosterone (14, 15, 17, 18). It is nowadays well established that adrenal steroids have steroid hormone cross-reactivity

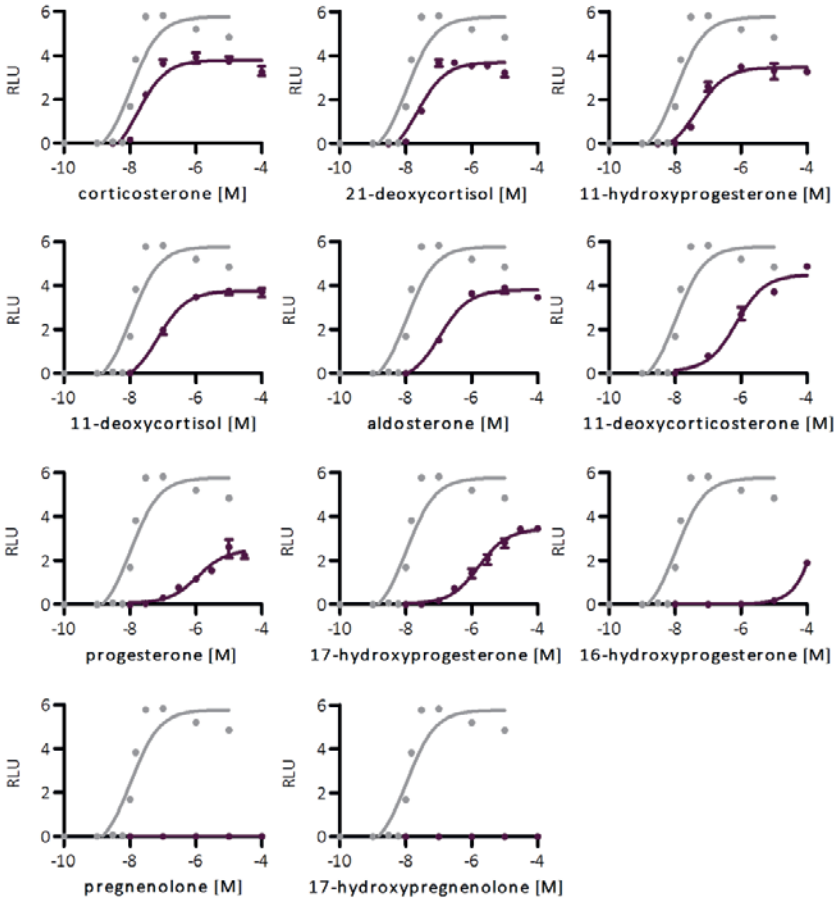


Fig. 3.3: Glucocorticoid receptor transactivation by adrenal steroid precursors. RLU is a measure for glucocorticoid receptor transactivation as measured by a dual-luciferase assay in human embryonic kidney cells that were exposed to increasing amount of steroids (depicted in black) for 24 hours. Cortisol was used as a reference (depicted in gray). All concentrations were measured in triplicate and mean and range are depicted. Abbreviations: RLU, relative light units.

explained by the high degree of homology of the DNA binding domain of the GR and the MR (19). Only one study showed that 21-deoxycortisol, 17-hydroxyprogesterone and progesterone are able to activate the GR (8). To the best of our knowledge, we are the first to describe the potency to activate the GR for more than 10 adrenal steroid precursors relative to cortisol. We found that 21-deoxycortisol and corticosterone had similar potency to activate the GR, while the potency of 11-hydroxyprogesterone, 11-deoxycortisol and aldosterone was 4-10x lower, and the potency of 11-

deoxycorticosterone, progesterone, and 17-hydroxyprogesterone more than 65x lower compared to cortisol.

Comparison of the transactivation data with the molecular structure of the adrenal steroid precursors shows that dehydrogenation of the steroid precursors by 3β-hydroxysteroid dehydrogenase is a prerequisite to enable a steroid to activate the GR, as pregnenolone and 17-hydroxypregnenolone, which are not dehydrogenated, were not able to cause GR transactivation *in vitro*. We also observed that dehydrogenation alone (progesterone) or combined with 21-hydroxylation (11-deoxycorticosterone) or 17-hydroxylation (17-hydroxyprogesterone) resulted in relatively low potency to activate the GR. Combination of dehydrogenation and hydroxylations of position 11 and 21 (corticosterone), or position 11 and 17 (21-deoxycortisol), or position 17 and 21 (11-deoxycortisol), increased the potency to activate the GR as EC_{50} values were similar or 7 times higher than of cortisol. Strikingly, the most potent GR activation precursor steroids are 11-hydroxylated steroids. This confirms the hypothesis of Hellal-Levy *et al.* (15) and Rousseau *et al.* (20) that hydroxylation at position 11 enhances glucocorticoid activity.

The strongly increased concentrations of the 11-hydroxylated steroid precursors 21-deoxycortisol and 11-hydroxyprogesterone in untreated 21OHD patients, that are able to activate the GR, might at least partially compensate for their cortisol deficiency. 17-hydroxyprogesterone and progesterone might also contribute as we found increased concentrations in 21OHD and 11OHD patients, although the potential to activate the GR is lower. Furthermore, in 11OHD patients, 11-deoxycortisol and 11-deoxycorticosterone also might compensate for the cortisol deficiency as these steroids had good GR activation potency.

A clinical threshold of 500 nmol cortisol/L blood after ACTH administration is widely used to define adrenal insufficiency, and although lower thresholds also have been proposed (9-11, 21), all of our patients had cortisol concentrations far below this threshold. Previously, it has been suggested to include serum corticosterone, 11-deoxycorticosterone and 11-deoxycortisol in addition to serum cortisol to evaluate adrenal function (22). Based on our finding of glucocorticoid receptor activation we emphasize the importance to use multiple adrenal steroid precursors, such as 11-deoxycortisol, 11-deoxycorticosterone, 11-hydroxyprogesterone, 21-deoxycortisol, 17-hydroxyprogesterone, progesterone, and cortisol to evaluate glucocorticoid action. More studies are necessary to study the clinical significance of our findings in more detail.

Although our data suggest that CAH patients may survive without glucocorticoid treatment, it has to be pointed out that based on our results we do not advocate to

withhold treatment in severely affected CAH patients. However, it has to be noted that in treated CAH patients blood concentrations of the elevated adrenal steroid precursors are generally suppressed and consequently patients become more dependent on glucocorticoid treatment and additional glucocorticoid stress dosing in the case of severe illness. As knowledge and infrastructure are far from perfect in developing countries, such as Indonesia, treated CAH patients may have an increased risk to develop adrenal crises as the compensatory effect of steroid precursors is absent. Also, acute discontinuation of glucocorticoid treatment, which is very likely in developing countries as medication is not always available, can increase the risk to develop adrenal crises as the compensatory steroid precursors will be suppressed for some time. Patients and clinicians should be aware of the importance of daily glucocorticoid supplementation without discontinuation in order to prevent adrenal crises. For the same reason, the importance of appropriate stress dosing regimen has to be emphasized.

Despite this being the first study in a relatively large cohort of untreated CAH patients, our study has some limitations. In some of the reported stress-situations, there may have been an underlying adrenal crisis as some episodes were reported as salt-wasting crises or severe vomiting and/or seizures. Still, it is remarkable that all patients recovered from these episodes without administration of glucocorticoid medication. Furthermore, none of our patients had the most severe type of CAH, a homozygous deletion of *CYP21A2*, although several patients were classified as genotype group 0, with an expected enzyme activity of <1%. As neonatal screening is not implemented in Indonesia, the prevalence of CAH patients is probably higher than the current number of patients diagnosed with CAH, which might have led to selection of our patient group. Especially male CAH patients are expected to be under-diagnosed, either because they have no complaints or because they have died within the first weeks of life due to a salt-wasting crisis. Education programs in these countries should therefore also focus on salt requirements in neonates to prevent salt-wasting crises.

In conclusion, our results show that severely affected untreated CAH patients with proven cortisol deficiency might survive due to accumulated adrenal steroid precursors which can activate the GR and might at least partially compensate for the cortisol deficiency. Especially 21-deoxycortisol, 11-hydroxyprogesterone, 11-deoxycortisol, and 17-hydroxyprogesterone can contribute to this compensation. Therefore, we suggest to measure all relevant GR activating steroids in the evaluation of adrenal function, especially in CAH patients. Further research should focus on establishing cut-off values for the combined adrenal steroid concentrations and to personalize treatment necessities according to the total panel.

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Disclosure Summary: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Supplemental Material:

LC-MS/MS protocol

Cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-hydroxyprogesterone, and progesterone were analyzed by LC-MS/MS after protein precipitation and solid-phase extraction. Internal standard [$^2H_4$]-cortisol, [$^2H_4$]-21-deoxycortisol, [$^2H_5$]-11-deoxycortisol, [$^{13}C_3$]-17-hydroxyprogesterone (Isosciences, King of Prussia, PA) and [$^2H_9$]-progesterone (CDN isotopes) was added to 100 µL serum. Subsequently 300 µL acetonitrile + 0.1% formic acid was added for protein precipitation. 300 µL H_2O was added to 200 µL supernatant followed by solid phase extraction (Oasis HLB 1cc, Waters). The eluate (methanol/isopropanol 95:5) was dried under a stream of N_2 gas, reconstituted in methanol: water (3:7) and injected (10 µL) into an Agilent Technologies 1290 Infinity UHPLC-system (Agilent Technologies, Santa Clara, CA) equipped with a BEH C18 (1.7µm 2.1 X 50mm) analytical column (Waters Corp.) at 60°C. Mobile phase A (methanol:water 20:80 + 2 mM NH_4CH_3COO + 0.1% formic acid) and B (methanol:water 98:2 + 2 mM NH_4CH_3COO + 0.1% formic acid) were run in a gradient (0.4 mL/min). Start gradient 70:30 A:B for 2.5 min; then to 40:60 A:B in 3.5 min; followed by a gradient in 0.5 min to 2:98 to remain such for 0.5 min and thereafter to 70:30 A:B in 0.5 min and remain such for 0.5 min. Retention time was 1.41 min, 2.13 min, 2.56 min, 4.66 min and 6.04 min for cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-hydroxyprogesterone and progesterone respectively. Total run time was 8 minutes. An 9-point calibration curve was used (cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-hydroxyprogesterone (Steraloids), progesterone (Sigma). An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in the electrospray positive ion mode, with a capillary voltage 3.5 kV, fragmentor voltage 380 V, sheath gas temperature 350°C and gas temperature 150 °C with N_2 collision gas. Two transitions (qualitative and quantitative) were monitored. Transitions (Q1>Q3) were m/z 363.4 > 97.1 (34 V) and m/z 363.4 > 121.1 (25 V) for cortisol; m/z 367.4 > 97.1 (34 V) and m/z 367.4 > 121.1 (25 V) for [$^2H_4$]-cortisol. m/z 347.2 > 121.1 (27 V) and m/z 347.2 > 269.0 (18 V) for 21-deoxycortisol; m/z 351.2 > 121.0 (29 V) and m/z 351.2 > 273.0 (18 V) for [$^2H_4$]-21-deoxycortisol. m/z 347.2 > 109.1 (31 V) and m/z 347.2 > 97.1 (29 V) for 11-deoxycortisol; m/z 352.3 > 113.1 (29 V) and m/z 352.3 > 100.1 (31 V) for [$^2H_5$]-11-deoxycortisol. m/z 331.3 > 109.1 (31 V) and m/z 331.3 > 97.1 (31 V) for 17-hydroxyprogesterone; m/z 334.3 > 112.1 (33 V) and m/z 334.3 > 100.1 (30 V) for [$^{13}C_3$]-17-hydroxyprogesterone. m/z 315.3 > 109.1 (29 V) and m/z 315.3 > 97.1 (29 V) for progesterone; m/z 324.3 > 113.1 (29 V) and m/z 324.3 > 100.1 (29 V) for [$^2H_9$]-progesterone. Dwell time was 100 ms, 20 ms, 20 ms, 60 ms and 100 ms for cortisol, 21-deoxycortisol, 11-deoxycortisol, 17-hydroxyprogesterone, and progesterone respectively. The method was linear assessed by CLSI EP6 protocol. For cortisol total CV

is 3.6% at 300 nmol/L and 3.1% at 1080 nmol/L. For 21-deoxycortisol total CV is 9.6% at 1.1 nmol/L and 8.6% at 15.2 nmol/L. For 11-deoxycortisol total CV is 5.9% at 2.1 nmol/L and 5.2% at 26.9 nmol/L. For 17-hydroxyprogesterone total CV is 5.6% at 2.6 nmol/L and 5.1% at 94.3 nmol/L. For progesterone total CV is 5.9% at 5.1 nmol/L and 3.9% at 30.4 nmol/L.

Corticosterone and 11-deoxycorticosterone were analyzed by LC-MS/MS after protein precipitation and solid-phase extraction. Internal standard [$^{2}H_4$]-corticosterone and [$^{13}C_3$]-11-deoxycorticosterone (Isosciences, King of Prussia, PA) was added to 100 µL serum. Subsequently 300 µL Acetonitrile + 0.1% formic acid was added for protein precipitation. 500 µL H_2O was added to 200 µL supernatant followed by solid phase extraction (Oasis HLB 1cc, Waters). The eluate (methanol/water 90:10) was dried under a stream of N_2 gas, reconstituted in methanol: water (3:7) and injected (10 µL) into an Agilent Technologies 1290 Infinity UHPLC-system (Agilent Technologies, Santa Clara, CA) equipped with a HSS T3 (1.8µm 2.1 X 100mm) analytical column (Waters Corp.) at 40°C. Mobile phase A (methanol:water 20:80 + 2 mM NH_4CH_3COO + 0.1% formic acid) and B (methanol:water 98:2 + 2 mM NH_4CH_3COO + 0.1% formic acid) were run in a gradient (0.4 mL/min). Start gradient 60:40 A:B for 3.5 min; then to 48:52 A:B in 1 min and 38:62 A:B in 3 min; followed by a gradient in 0.01 min to 5:95 A:B to remain such for 1 min and thereafter to 60:40 A:B in 0.5 min and remain such for 2 min. Retention time was 5.6 min and 7.2 min for corticosterone and 11-deoxycorticosterone, respectively. Total run time was 11 minutes. A 9-point calibration curve was used (corticosterone and 11-deoxycorticosterone (Sigma)). An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in the electrospray positive ion mode, with a capillary voltage 3.0 kV, fragmentor voltage 380 V, sheath gas temperature 350°C and gas temperature 150 °C with N_2 collision gas. Two transitions (qualitative and quantitative) were monitored. Transitions (Q1>Q3) were m/z 347.2 > 97.0 (31 V) and m/z 347.2 > 121.1 (29 V) for corticosterone; m/z 351.3 > 97.1 (31 V) and m/z 351.3 > 121.1 (29 V) for [$^{2}H_4$]-corticosterone; m/z 331.3 > 109.1 (31 V) and m/z 331.3 > 97.1 (29 V) for 11-deoxycorticosterone; m/z 334.3 > 112.2 (29 V) and m/z 334.3 > 100.2 (27 V) for [$^{13}C_3$]-11-deoxycorticosterone. Dwell time was 100 ms for both corticosterone and 11-deoxycorticosterone. The method was linear assessed by CLSI EP6 protocol. For corticosterone total CV is 3.2% at 26.8 nmol/L and 3.6% at 59.4 nmol/L. For 11-deoxycorticosterone total CV is 3.7% at 0.2 nmol/L and 2.7% at 2.0 nmol/L.

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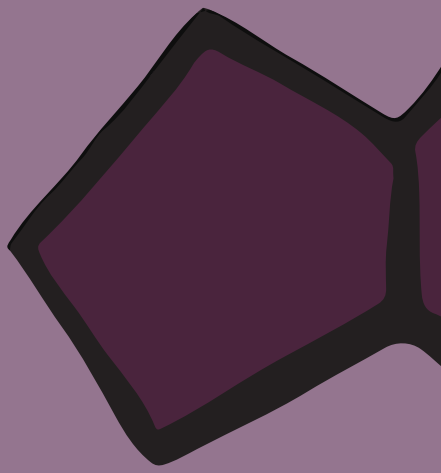

Chapter 4

Testicular adrenal rest tumors: current insights on prevalence, characteristics, origin and treatment

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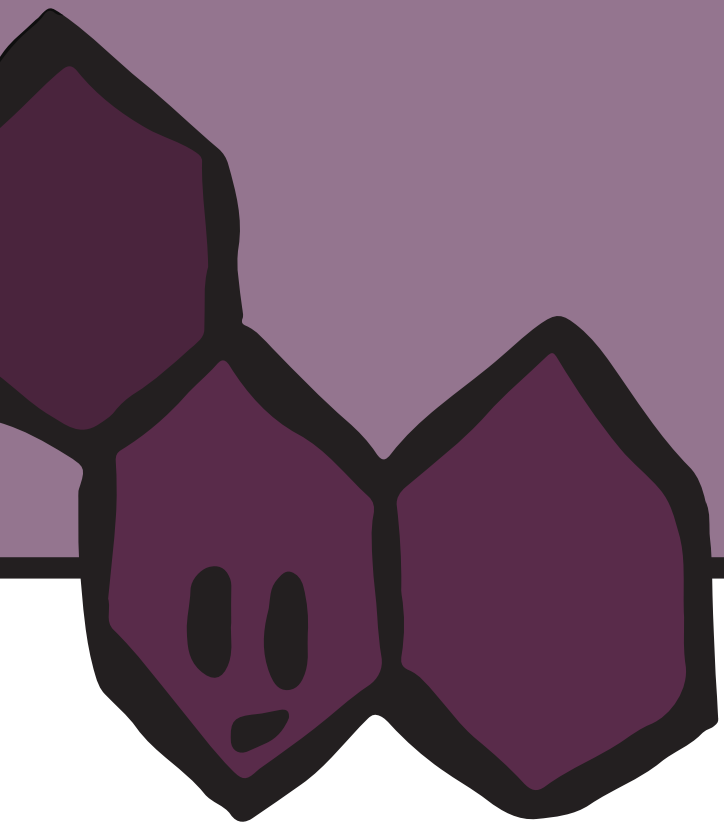

Abstract

This review provides the reader with current insights on testicular adrenal rest tumors (TARTs), a common complication in male patients with congenital adrenal hyperplasia (CAH), often leading to sub- or infertility. Presently, more than 300 individual cases of CAH-associated TART have been reported since the first description in 1940. In recent studies, an overall prevalence of 40% (range 14-89%) is found in classic CAH patients. Reported differences in prevalence numbers are mainly caused by the method of detection (palpation vs. ultrasound or MRI), and the selected patient population (child vs. adult, classic vs. non-classic CAH). Biochemically, histologically, and molecularly TARTs exhibit particular adrenal characteristics and were therefore thought to originate from aberrant adrenal cells. More recently TART has been found to also exhibit testicular characteristics. This has led to the hypothesis of a pluripotent cell type as the origin of TART. High concentrations of adrenocorticotropic hormone (ACTH) could cause hyperplasia of these pluripotent cells, as TART appears to be associated with poor hormonal control with concomitant high ACTH concentrations. Unfortunately, as yet there are no treatment options to prevent the development of TART, nor are there guidelines to treat patients with TART. Intensified glucocorticoid treatment could improve fertility status, although studies report contradicting results. As TART can lead to irreversible testicular damage, semen cryopreservation could be offered to patients with TART. Further research should focus on druggable targets to prevent the development of TART or to treat TART and improve fertility status of patients with TART.

Introduction

Congenital adrenal hyperplasia (CAH), a group of autosomal recessive disorders of the adrenal cortex affecting adrenal steroid production, is caused by a defect of one of the enzymes involved in the adrenal steroidogenesis. In most (>90%) CAH patients the enzyme *CYP21A2* (21-hydroxylase) is affected. The incidence is 1:12 000 (1, 2). The severity of the disease depends on the residual enzyme activity which depends mainly on the genotype. Patients classified as genotype null (0) or A have <1% residual enzyme activity with cortisol and aldosterone deficiency (also classified as classic salt-wasting type). Patients classified as group B genotype have 1-5% residual enzyme activity with cortisol deficiency but in general sufficient (or mildly deficient) mineralocorticoids (classic simple virilizing type). Patients in group C have a residual enzyme activity of 20-50% with a less severe phenotype of mildly increased androgens and are considered as non-classic CAH (3).

In CAH, cortisol production is decreased due to the enzymatic defect and, consequently, pituitary adrenocorticotropic hormone (ACTH) production is increased due to the lack of negative feedback of cortisol to the pituitary gland. This causes hyperplasia of the adrenal gland and stimulation of adrenal steroidogenesis, resulting in accumulation of steroids before the enzymatic block. These steroids are subsequently shunted to the unaffected androgen pathway, leading to prenatal virilization of the female external genitalia. Therapy consists of lifelong glucocorticoid administration to substitute the cortisol deficiency, thereby also restoring the negative feedback on the pituitary gland. This results in lowering ACTH and adrenal androgens concentrations. However, supraphysiological dosages of glucocorticoids are often needed to adequately suppress adrenal androgen production (1, 2).

Subfertility is a severe long-term complication in adult CAH patients (4-13), the main cause of which is the presence of testicular adrenal rest tumors (TARTs). TARTs are benign lesions within the testes that may cause infertility due to obstruction of the seminiferous tubules (14) or due to paracrine effects of hormones produced by TART (15-18). Longstanding TART can lead to irreversible damage of the testicular tissue (14). Therefore, knowledge about the prevalence, pathophysiology and treatment of TART in CAH patients is of major clinical importance. In this review we provide an overview on current insights in TART in males with CAH due to 21-hydroxylase deficiency, including prevalence, characteristics, origin, and treatment options.

Prevalence of TART

Since the first description of TART in 1940 by Wilkins *et al.* (19) many studies described the prevalence of TART mainly in case reports or small cohorts. In Fig. 4.1, we summarize 31 original studies that have reported on TART prevalence in the last ten years using ultrasound or MRI (7, 8, 10, 11, 13, 20-45). Reported prevalence ranged from 14% to 86%, depending on age and genotype. When summed, the overall TART prevalence in the reported CAH patients in the last ten years was 38% (472/1251).

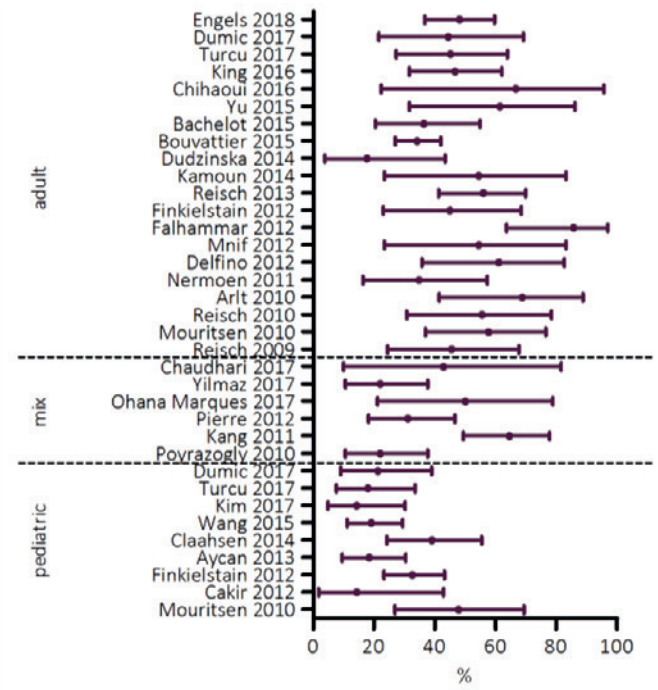

Fig. 4.1: Prevalence of TART. Reported prevalence of TART within the last ten years. Age classification (pediatric, mix, or adult) of the patients was used a defined by the original author of the study. The black dots indicate the prevalence of TART reported and the whiskers indicate the 95% confidence intervals.

Method of detection

Due to its central location within the rete testis, TART can only be detected by palpation if the lesion has a diameter of at least two centimeters. MRI and ultrasound enable the detection of smaller lesions, even of several millimeters (Fig. 4.2). This explains why higher prevalence numbers of TART are found in MRI or ultrasound

studies compared to studies using palpation as method of detection (8, 10, 21, 24-28, 30, 32, 35, 37-41). Ultrasound is the preferred method of detection as it is easy accessible and has a good sensitivity to detect small lesions.

Age

The broad prevalence range can be partially explained by the variation in patient age. Studies in adult CAH patients (defined either as 16+ or 18+) showed a higher prevalence of TART (summed prevalence 46%, N=296/642) compared to the studies only including children (summed prevalence 25%, N=104/416) (Fig. 4.1). Concordantly, some studies also reported an increase in prevalence with age, especially during puberty (21, 25, 30, 35, 45). The youngest patient described in the last ten years was 1.8 years old (21). TARTs have also been previously reported in autopsy material of CAH patients less than eight weeks old (46).

Severity of the CAH

Several studies describe an association between the prevalence of TART and the severity of the disease. In the last ten years a total of 1019 classic CAH males were studied in 27 studies, of whom 40% (range 14 - 89%) had TART (n=412) (7, 8, 10, 11, 13, 21, 22, 24-27, 29-39, 41-45). Overall, prevalence of TART in the most severely affected CAH patients (genotype 0 and A, and salt-wasting type) is 51% (228/451) (7, 11, 13, 21, 22, 24-27, 30, 32-35, 37-39, 41, 42, 44, 45), while this is 23% (53/227) for the less severely affected classic CAH patients (genotype B and simple virilizing type) (7, 13, 21, 22, 24, 25, 27, 30, 32-35, 37-39, 41, 42, 44, 45). Within reported classic CAH patients with TART, 80% (228/285) were severely affected (genotype 0 and A, salt-wasting type), and only 20% (57/285) were less severely affected (genotype B, simple virilizing type), indicating that TART is more prevalent in more severely affected CAH patients. Nor genotype nor phenotype were significantly associated with TART in five studies (11, 30, 36, 38, 42), although this may have been caused by low patient numbers and subsequent low statistical power.

Non-classic CAH patients were included in six studies (21, 25, 29, 30, 36, 45). In none of the 37 patients TART was present on ultrasound. In contrast, Falhammar *et al*. reported two patients with non-classic CAH (mildest mutation V281L and P105L+P453S), who both had TART (38), but the phenotype was not described in detail. Although the majority of patients with a V281L mutation has non-classic CAH, some present with a more severe phenotype (47). Moreover, the combination of P105L and P453S mutations are synergistic, resulting in more than 90% reduction of enzyme activity *in vitro* (48). Therefore, these patients might be classified as genotype B (more affected classic form) according to the classification used by Krone *et al.*(3). We earlier

described (13) two patients with non-classic CAH with TART. Mutation analysis in both showed deletion/P30L mutation. However, the P30L mutation can be classified as genotype B or genotype C according to the classification used by Krone *et al.* (3). These patients might therefore also belong to the more affected classic form of CAH patients.

In conclusion, the prevalence of TART in CAH patients is almost 50% and associated with method of detection, age, and severity of the disease.

Characteristics of TART

Ultrasound and MRI features of TART

Currently, TARTs are mostly detected using ultrasound or MRI techniques (Fig. 4.2). On ultrasound imaging, the majority of TARTs (83%) were reported as hypoechogenic (220/266), 7% of the TARTs (18/266) were described as hypoechogenic with hyperechogenic reflections, and only 6% (17/266) were described as hyperechogenic. The remaining 4% was described as isogenic (6/266), disseminated (3/266) or not specified (2/266) (21, 23, 26, 28, 30, 32, 35, 40, 41, 49-55). Hypervascularity is common in TART and can be detected using color Doppler ultrasound (23, 26, 28, 35, 49, 53, 54). In MRI imaging TART shows hypointensity on T2 images, while TARTs appear hyperintense or isointense on T1 images compared to the normal testicular parenchyma (23, 53, 54, 56).

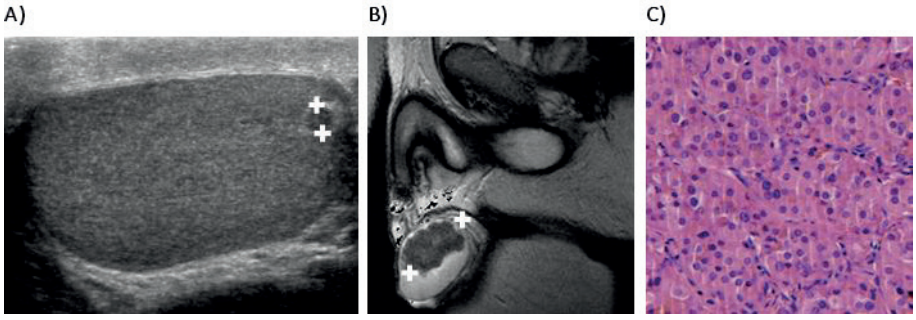

Fig. 4.2: Ultrasound (A) and MRI (B) images and H&E staining (C) of testicular adrenal rest tumor (TART). **A)** Scrotal ultrasound of a 19-year old male CAH patients. The transverse image shows a hypoechogenic rounded lesion (between the white indicators) in the left testis. **B)** T2-weighted MR image of longstanding bilateral TARTs in a 33-year old patient. Heterogeneous low-signal intensity tumors (between the white indicators) are displacing surrounding high signal normal testicular tissue. **C)** TART tissue exist of large polygonal cells with abundant eosinophilic cytoplasm. Original magnification used 10x.

Macroscopic and microscopic features

TARTs are typically located near or within the mediastinum testis (6, 23, 28, 30, 32, 35, 39, 42, 45, 49, 51-53, 57-59) with bilateral presence reported in 33 studies (6, 7, 10, 13, 21-28, 30, 32, 34, 35, 38, 40-42, 44, 45, 49-57, 60, 61) and an overall percentage of 77 (339/441). Macroscopically, TARTs are described as firm (5, 14, 49, 56-59, 61-63), yellow to tan colored tumors (14, 57-59, 61-63), which are round to oval (23, 28, 30, 40, 42, 51) or multilobular (14, 57, 58, 62, 63). They are sharply delineated from normal testicular tissue (14, 16, 23, 28, 52, 54, 56, 57, 59, 62-64). Microscopically (Fig. 4.2C), TARTs contain large polygonal cells with abundant eosinophilic cytoplasm (14, 16, 57-59, 61, 63) and round nuclei (14, 57, 59, 61). These features are consistent with steroid secreting cells, such as Leydig cells (59, 61, 65, 66). Sheets, cords or nests of hyperplastic cells are separated by bands of fibrous tissue (14, 57-59, 61-63, 65), which explains the firm rubbery appearance of TART. Some TART nodules even appear calcified (61) or have severe hyalinization (14).

Differentiation of TART from Leydig cell tumors

Pathologically, it is challenging to discriminate TART from Leydig cell tumors (LCTs) as these tumors are closely related and share morphological characteristics. Discrimination of these tumors is of importance as misdiagnosis can lead to incorrect treatment (67, 68). Currently, TART is surgically removed only when severe pain complaints are present (58), while LCTs are always removed either with testis-sparing surgery or orchiectomy, as 10% of these tumors are malignant (66).

Few characteristics are used to discriminate between TART and LCT, although none of them is exclusive for one of the tumor types. CAH patients are more likely to have TART, however few cases of LCT in CAH patients have been reported (69-74). Furthermore, bilateralism is reported in 77% of the CAH patients with TART (summarized in this review), while unilateralism is found in >90% of the patients with LCT (66). Imaging and biopsy of the tumor cannot discriminate between the different tumor types as these both show (steroidogenic) masses. TART size can decrease after intensifying glucocorticoid treatment of which a few cases are known (5, 59, 75-79). However, not all TARTs respond to increased glucocorticoid treatment especially in longstanding TART when fibrosis of the tumor is present (5, 50, 56, 59, 61, 75-79). Until now, no single marker that can discriminate TART from LCT is known, which can lead to misdiagnosis and consequently incorrect treatment. Therefore, there is a need for better discriminative markers.

Gene expression

Gene expression analyses in TART have been reported in four studies (62, 63, 65, 80). Expression of adrenal specific genes, such as *CYP11B1* (62, 63, 65)*, CYP11B2* (62, 63)*, CYP21A2* (65), and *DLK1* (65), were found in TART. Moreover, gene expression of the -adrenal- ACTH (*MC2R* (62, 63, 65)) and angiotensin II receptor (*AGTR2* (62, 63)) were reported. Gene transcripts of enzymes of common pathways in the steroidogenesis (testes and adrenal), *i.e. STAR* (62), *CYP11A1* (62, 65), *CYP17A1* (62), *HSD3B2* (62), *NR5A1* (62) were also described. GATA transcription factors are involved in adrenogonadal development (81). We found *GATA3* and *GATA6* expression in TART and adrenal tissues, while *GATA4* was expressed in TART and testicular tissues (80). Interestingly, in another study we also found testis-specific gene expression of the luteinizing hormone (LH) receptor (*LHCGR)*, *INSL3*, and *HSD17B3* (62). However, gene expression of *INSL3* was not confirmed by Lottrup *et al.* (65). An overview of all reported gene expression analyses in TART is given in Table 4.1. To summarize, both adrenal- and testis-specific gene expression has been found in TART.

Immunohistochemistry

Eight studies investigated the expression of multiple proteins with immunohistochemistry in TART tissues of CAH patients (14, 28, 50, 57, 59, 61, 65, 82), while four additional studies reported on the expression of insulin-like 3 (62), inhibin A (27), Reinke crystals (63), or POU domain class 5 transcription factor 1 (83). An overview of these findings is given in Table 4.1. As described before, TART is morphologically closely related to LCT, a sex-cord-stromal tumor. Several proteins specific for sex-cord-stromal tumors were investigated to find immunophenotypic differences between TART and LCT. Epithelial membrane antigen and paired box 2 and paired box 8 were negative in normal Leydig cells, LCT, TART, and normal adrenal tissues (82). Protein S100 showed variable expression in normal Leydig cells, but was negative in LCT, TART, and adrenal tissues, while stem cell growth factor receptor c-kit was variably expressed in LCT, but negative in normal Leydig cells, TART and adrenal tissues (82). However, vimentin staining was reported positive in TART in two studies (28, 57) and variable in one study (82), whereas positive staining was also observed in normal Leydig cells, LCT and the zona glomerulosa of the adrenal gland (82). Synaptophysin also showed variable expression with weak (82) to strong (50, 57) intensity in TART, while expression in LCT was reported as variable with weak to moderate intensity (50, 57, 82). Variable staining with weak (82) to strong (50) intensity can be observed in adrenocortical tissue, while staining in normal Leydig cells was rarely seen (50, 82). Neural cell adhesion molecule 1 was the only sex-cord-stromal tumor maker reported positive in TART in 3 studies (28, 57, 82) whereas positive staining was also observed in LCT (57, 82), normal Leydig cells and the zona

glomerulosa (82). To summarize, proteins specific for sex-cord-stromal tumors were in general negative in TART. Vimentin, synaptophysin and neural cell adhesion molecule 1 were expressed variably in TART and could not discriminate TART from LCT.

As TART is known to have adrenal characteristics several studies also studied protein expression of adrenal markers. Inhibin A staining is used as an adrenocortical marker (zona reticularis) and was positively expressed in TART (27, 28, 50, 57, 82), although positive staining was also found in Leydig cells and LCTs (50, 82). Another adrenal marker, protein delta homolog 1 (from DLK1), was expressed in TART, but almost absent in LCT (65). Chromogranin, a neuro-endocrine marker, was absent in TART (57, 82), Leydig cells, LCTs and adrenocortical cells (82). Ki67, a proliferation marker that is correlated with the clinical course of neuro-endocrine tumors, was found variably expressed and low staining intensity was observed in both TART and LCT (57). Smooth muscle actin, a marker for vascularization, was positive in TART, indicating prominent vascularization in TART (65). Lipochrome consists of golden-brown lipofuscin pigments, which occur in the zona reticularis of the adrenal gland and testis interstitium and was observed in TART (14, 16, 50, 57, 59, 61), but not in LCT tissue (57). In addition, the ACTH receptor (65) and the angiotensin II receptor (16) were observed in TART. To summarize, adrenal markers and receptors are expressed in TART, while neuro-endocrine markers are absent.

Furthermore, the occurrence of androgen producing cells have been investigated in TART via specific protein markers. Immunohistochemistry of insulin-like 3, a marker of Leydig cells, showed negative staining in TART (62, 65). Reinke crystals, another marker for Leydig cells and a typical finding in LCT were also absent in TART (14, 16, 50, 54, 57, 59, 61, 63, 82). The androgen receptor was found variably expressed (82) or not expressed (57). LH binding was not observed in a study which examined one TART tissue (16). Calretinin a marker for androgen producing cells was variably expressed in TART (82). Keratin, another marker for androgen producing cells, was positively (57) or negatively (82) expressed in TART. Cholesterol side-chain cleavage enzyme (65), and 17α-hydroxylase (82) are expressed in TART and normal Leydig cells. Variable expression of the testicular enzyme 17β-hydroxysteroid dehydrogenase was observed (82). Moreover, enzymes from the adrenal steroidogenesis were studied: 21-hydroxylase (65), cytochrome P450 11B1 (16, 65), and 3β-hydroxysteroid dehydrogenase (16, 65, 82) were all expressed in TART tissue, but not in normal testicular tissue. POU domain, class 5 transcription factor 1 (OCT4) is a protein expressed in undifferentiated, pluripotent cells, but was not expressed in TART or LCT (83). To summarize, markers for androgen producing cells were expressed in TART, but specific markers for Leydig cells were absent.

Overall, immunohistochemistry results show that proteins routinely investigated in sex-cord-stromal tumors are in general absent in TART, but TART tissue does express markers for androgen-producing cells. Also, adrenocortical markers as well as proteins from the adrenal steroidogenesis are expressed in TART.

Table 4.2: Expression of markers in testicular adrenal rest tumor tissue.

Marker	Present in TART	Variable in TART	Absent in TART
Adrenal gene expression			
CYP11B1	(62, 63, 65)		
CYP11B2	(62, 63)		
CYP21A2	(65)		
DLK1	(65)		
MC2R	(62, 63, 65)		
AGTR2	(62, 63)		
GATA3	(80)		
GATA6	(80)		
Gene transcript of common enzymes in steroidogenesis			
STAR	(62)		
CYP11A1	(62, 65)		
CYP17A1	(62)		
HSD3B2	(62)		
NR5A1	(62)		
Testicular gene expression			
GATA4	(80)		
LHCGR	(62)		
INSL3	(62)		(65)
HSD17B3	(62)		
Proteins specific for sex-cord-stromal tumors			
Epithelial membrane antigen			(82)
Paired box 2			(82)
Paired box 8			(82)
S100			(82)
Stem cell growth factor receptor c-kit			(82)
Vimentin	(28, 57)	(82)	
Synaptophysin		(50, 57, 82)	
Neural cell adhesion molecule 1	(28, 57, 82)		
Adrenal markers			
Inhibin A	(27, 28, 50, 57, 82)		
Protein delta homolog 1 (from DLK1)	(65)		
Neuro-endocrine markers			
Chromogranin			(57, 82)
Ki67		(57)	
Marker for vasculature			
Smooth muscle actin	(65)		
Pigments			
Lipochrome	(14, 16, 50, 57, 59, 61)		

Receptors			
ACTH receptor	(65)		
Angiotensin II receptor	(16)		
Androgen producing cell markers			
Insulin-like 3			(62, 65)
Reinke crystals			(14, 16, 50, 54, 57, 59, 61, 63, 82)
Androgen receptor		(82)	(57)
Calretinin		(82)	
(Pan)keratin	(57)		(82)
Cholesterol side-chain cleavage enzyme	(65)		
17α-hydroxylase	(82)		
Testicular enzyme			
17β-hydroxysteroid dehydrogenase		(82)	
Adrenal steroidogenesis enzymes			
21-hydroxylase	(65)		
Cytochrome P450 11B1	(16, 65)		
3β-hydroxysteroid dehydrogenase	(16, 65, 82)		
Marker for undifferentiated cells			
POU domain, class 5 transcription factor 1 (from OCT4)			(83)

Abbreviations: TART, testicular adrenal rest tumor.

Steroid production

CAH patients with TART had higher concentrations of androstenedione, 17-hydroxyprogesterone, and 21-deoxycortisol in spermatic vein blood compared to peripheral blood (63), indicating steroid production by TART. Especially 21-deoxycortisol production is of importance as this is an adrenal-specific steroid hormone (11-hydroxylated) and not produced by the testis. Furthermore, Turcu *et al.* (84) showed that 17-hydroxyprogesterone, 21-deoxycortisol, 16-hydroxyprogesterone, and progesterone were the most abundant steroids produced by TART cells *in vitro*.

Using immunohistochemistry, Mesa *et al.* found TART tissue to express steroid hormones dehydroepiandrosterone, androstenedione, and testosterone (82), which reflects the overflow of precursor steroids into the androgen pathway. Turcu *et al.* (22) determined steroid concentrations in serum of CAH patients with TART compared to the concentrations in CAH patients without TART. 11-hydroxyandrostenedione, 11β-hydroxytestosterone, 11-ketotestosterone, 11-ketoandrostenedione, pregnenolone sulfate, 17-hydroxypregnenolone sulfate, androsterone, allopregnanolone, and androstenedione were significantly higher in CAH patients with TART compared to CAH patients without TART indicating higher steroid production in CAH patients with TART

possibly by the TART tissue itself or associated with undertreatment of the CAH patient resulting in less androgen suppression (22).

To summarize, TART is mostly present in both testes, typically located within the rete testes and difficult to discriminate from LCT. Several studies have characterized TART tissue and found that protein expression in TART shows similarity with normal and aberrant Leydig cells (LCT), as well as adrenocortical protein expression. This is in line with the published results on gene expression data, indicating both adrenal and testicular characteristics. Furthermore, gene and protein expression of adrenal-specific enzymes together with the measured steroids implicate that TARTs are able to produce steroid hormones.

Characteristics of CAH patients with TART

Association between hormonal control of CAH and the presence of TART

Poor hormonal control was reported in more than half of the studied CAH patients with TART (55/95) (21, 23, 27, 30, 32, 34, 38, 39, 45, 52, 56, 60, 85). Only two studies reported no clear association between (poor) hormonal control and the presence of TART (7, 29). Poor hormonal control is biochemically characterized by elevated ACTH concentrations and consequently (strongly) elevated 17-hydroxyprogesterone and androstenedione concentrations. Higher serum ACTH concentrations were reported in CAH patients with TART compared to CAH patients without TART (27, 40, 41, 45). However, two studies found no significant association between TART and ACTH concentrations (22, 29). Higher concentrations of 17-hydroxyprogesterone were reported in CAH patients with TART compared to those without TART (13, 41, 45, 52, 57, 60), although, again, several other studies did not find an association (25, 27, 29, 33, 35). Androstenedione concentrations were found to be higher (13, 36, 41, 57, 60), or similar (25, 29, 35) in CAH patients with TART compared to CAH patients without TART. Signs of long-term poor hormonal control were reported in the majority of the CAH patients with TART, such as reduced final height and advanced bone age in children (64%: 25/39 (21, 25, 51, 55)). However, advanced bone age was also found in CAH patients without TART and the area under the curve of the difference between bone age and chronological age over the years did not differ between CAH patients with and without TART (33). Lower final adult height in CAH patients with TART was found compared to those without TART by Kang *et al.* (42). Reisch *et al.* showed that TART volume was not associated with any marker of hormonal control (7). The absence of such association might be explained by the fact that the TART size in longstanding

TART is mostly due to fibrosis or hyalinization instead of presence of hyperplastic TART cells (86).

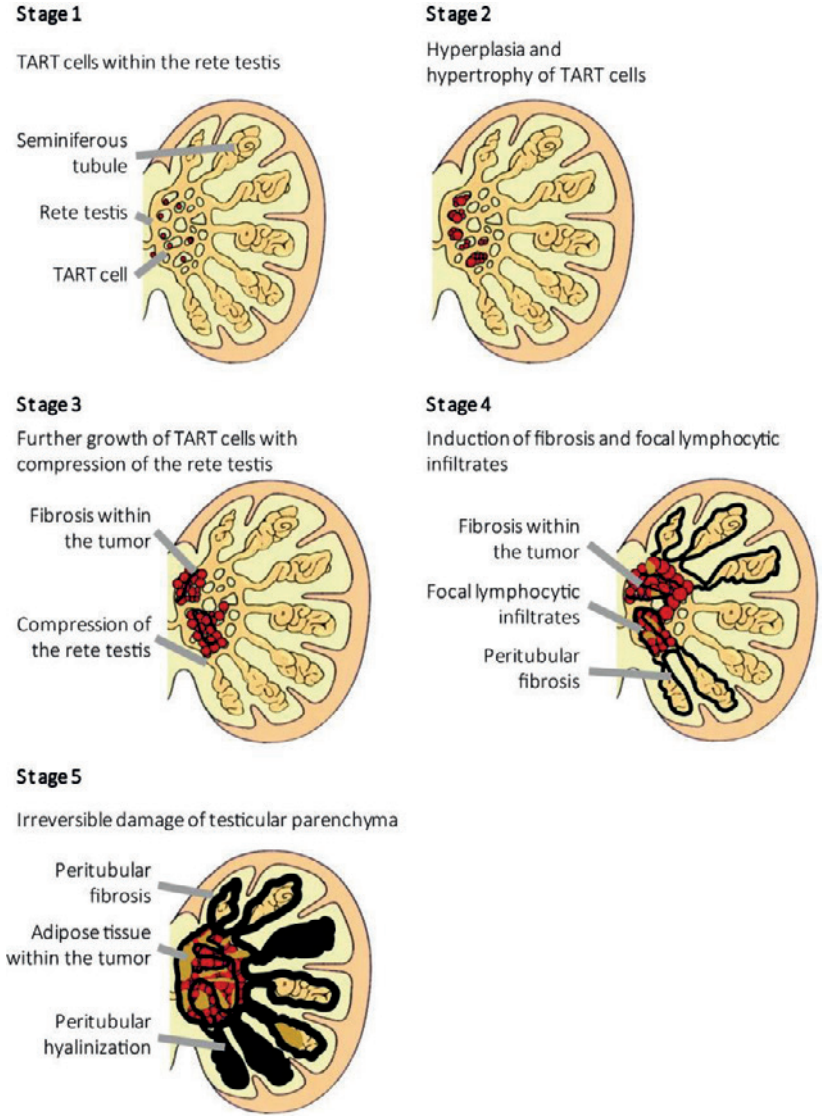

Fig. 4.3: Development of testicular adrenal rest tumor (TART) and proposed staging of Claahsen *et al.* (86). Stage 1 indicates the presence of individual TART cells within the rete testis. These cells become hyperplastic and hypertrophic (stage 2). Further growth of the TART cells will lead to obstruction of the seminiferous tubules, causing sub- or infertility (stage 3). In longstanding TART, progressive obstruction of the rete testis may lead to induction of fibrosis within TART and focal lymphocytic infiltrate. Peritubular fibrosis in the surrounding testicular tissue indicates early testicular damage (stage 4). The chronic obstruction will lead to hyalinization causing irreversible damaged testicular parenchyma (stage 5).

CAH patients receive lifelong substitution therapy to restore the hormonal balance. Glucocorticoids are used to treat cortisol deficiency and consequently lower ACTH concentration. Remarkably, no differences between glucocorticoid dosages were found between CAH patients with and without TART (22, 25, 27, 29, 33, 35, 36, 41). A possible explanation for this might be noncompliance, as prescribed glucocorticoid dosages do not always reflect intake of the medications. In contrast, CAH patients with TART had higher prescribed fludrocortisone dosages compared to CAH patients without TART (25, 29, 35, 41) reflecting a more severe salt-wasting form of CAH or noncompliance, although interpretation of these results is troublesome as only limited data is available.

Long-term consequences of TART

It is thought that TARTs develop from single precursors cells into large (multinodular) masses within the rete testis (Fig. 4.3). Claahsen *et al.* proposed to use 5 stages to classify TART (86), starting with individual TART cells (stage 1) that become hyperplastic and hypertrophic (stage 2). Further growth of the TART cells will lead to obstruction of the seminiferous tubules, causing sub- or infertility (stage 3). In longstanding TART, fibrosis within TART increases, focal lymphocytic infiltrate and peritubular fibrosis (stage 4) and eventually hyalinization (stage 5) develops causing irreversible damaged testicular parenchyma, leading to azoospermia (14). Due to compression of the testicular parenchyma, longstanding TART can cause severe pain complaints (58).

To summarize, poor hormonal control with elevated ACTH concentrations seems to be associated with TART, although contradicting results are reported. Furthermore, TART can cause severe pain complaints and irreversible damage to the testicular parenchyma, leading to infertility.

Origin and development of TART

Only a few studies have speculated on the origin of TART. Originally, it was hypothesized that during adrenogonadal development *in utero* aberrant adrenal cells end up within the testes, which usually show spontaneous regression within the first year of life. In CAH patients, these adrenal rest cells may proliferate in the presence of increased concentrations of growth promoting factors, such as ACTH. This hypothesis was strengthened by the fact that several adrenal-specific characteristics were found in TART cells, as described in the section above. Nowadays the hypothesis on the origin of TART has shifted. Recently, testicular characteristics were also found in TART, as shown by gene expression of *LHCGR*, *INSL3*, and *HSD17B3* (62) and *GATA* transcription factors (80) and the presence of the testicular enzyme 17β-hydroxysteroid dehydrogenase

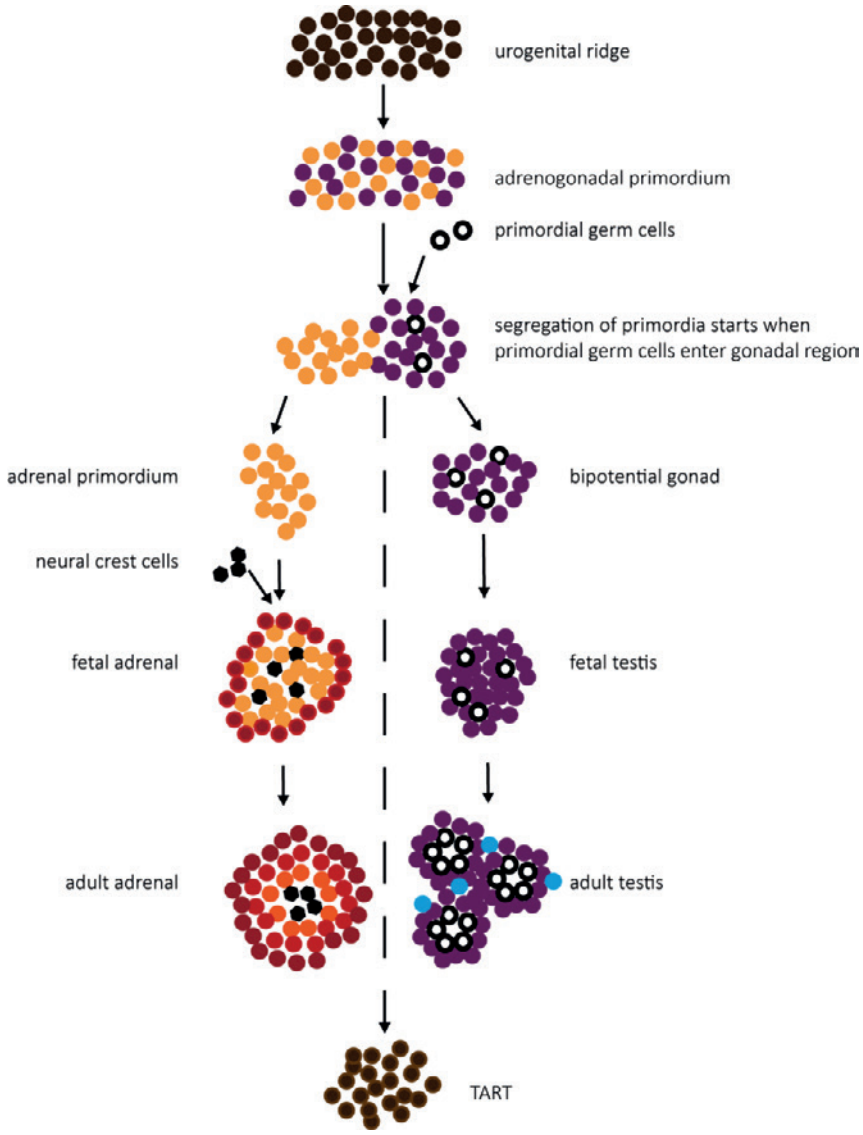

Fig. 4.4: Adrenogonadal development. The adrenal glands and testes originate from the urogenital ridge of which the adrenogonadal primordium is formed. Segregation of this primordium into an adrenal primordium and bipotential gonad starts when primordial germ cells enter the gonadal region of the adrenogonadal primordium. The fetal adrenal exist of an medulla formed by neural crest cells, a fetal cortex and a definitive cortex surrounding the fetal cortex. The fetal cortex regresses after birth and the definitive cortex develops in the adult cortex containing three zones: zona glomerulosa, zona fasciculata and zona reticularis. The fetal testis develops from the bipotential gonad and somatic cells completely invert germ cells forming testicular cords. The adult testis exist of seminiferous tubules and Leydig cells (104). TART exhibit both testicular and adrenal characteristics and might therefore originate from cells of the urogenital ridge or adrenogonadal primordium as this is the common precursors for both the testis and the adrenal glands (indicated by the dashed line).

(82). This indicates that TART has both adrenal as well as testicular characteristics and the capacity to produce adrenal specific hormones. We therefore hypothesize that TART originates from a more pluripotent cell type that is already present *in utero*, probably originating from the adrenogonadal primordium or from the urogenital ridge (Fig. 4.4).

Fetal Leydig cells or adult Leydig cell precursors might be good candidates (87). In contrast to the adult Leydig cell the fetal Leydig cell has both Leydig and adrenocortical cells features, *i.e.* fetal Leydig cells contain ACTH and LH receptors and can be stimulated by pituitary ACTH and LH. In addition, the adrenocortical specific steroidogenic enzyme (*CYP11B1*) is expressed in fetal mouse testes. The adrenal-like cells in the developing mouse testes described by Val *et al.* (88) have the same characteristics, which therefore might also be good candidates for the origin of TART or may be consistent with the fetal Leydig cell. TART is not found in deceased non-CAH neonates by histological examination (89), which indicates that either the pluripotent cells are not present in healthy subjects or that they lack the fetal stimulus for the outgrow of these cells.

We propose that ACTH is the most important stimulating factor causing the hyperplasia of pluripotent cells to TART, as ACTH receptors are found in TART tissues and poor hormonal control with elevated ACTH concentrations seem to be associated with increased TART prevalence. The higher prevalence of TART in the most severe forms of CAH and expected higher ACTH concentrations already *in utero* and the low prevalence in non-classic CAH with only moderate ACTH elevations supports this hypothesis. Furthermore, TART has been described in conditions with strongly elevated ACTH concentrations, such as Cushing's syndrome (50, 90, 91), Nelson's syndrome (90-93), and Addison's disease (50), illustrating the importance of early (childhood) and longstanding exposure to elevated ACTH concentrations in the development of TART. Interestingly, TART was not observed in adult male patients with high ACTH concentrations due to acquired Addison disease or bilateral adrenalectomy (94). This might suggest that early exposure already *in utero* or young childhood to ACTH is necessary to stimulate proliferation of pluripotent cells into multiple small tumors which confluents to a multinodular lesion over time. The further growth of TART may depend on the cumulative exposure (duration and concentration) to ACTH and probably also angiotensin II (16, 62, 63), as also angiotensin II receptors are present in TART. The pubertal rise of LH may also contribute to further proliferation of these aberrant cells as LH receptors have been found in TART (21, 25, 30, 35, 45, 62).

Recently, we described that particular precursor steroids accumulate before the enzymatic defect in CAH. These precursor steroids, 21-deoxycortisol, 17-hydroxyprogesterone, and progesterone, exhibit glucocorticoid activity and because of

their increased levels are able to compensate at least partially for the glucocorticoid deficiency in CAH patients (95). It is unclear whether these steroids, which do not occur in appreciable concentrations in non-CAH patients, have any role in the development of TART.

To summarize, TART originates from still unknown pluripotent cells of the adrenogonadal primordium or urogenital ridge. Elevated ACTH seems to be the most important stimulating factor for outgrow and hyperplasia of these pluripotent cells. However, TART prevalence is only 40% in classic CAH patients, whereas all these patients experience high ACTH concentrations. This might indicate the importance of initiation of the ACTH stimulus. Pluripotent cells seem to require a prenatal stimulus to prevent regression of these cells, in which the timing of increased ACTH concentration might play an important role. Further research is needed to establish the role of initiation, duration of exposure and concentrations of ACTH on TART development. Also, the role of other possible stimulating factors, such as LH and angiotensin II, should be established.

Treatment of TART

Until now, no clear guidelines exist to treat or prevent development of TART (2, 96). TART treatment is mostly focused on restoring fertility in adult patients. The first choice of treatment is intensifying glucocorticoid treatment to suppress ACTH, especially in poorly controlled CAH patients. However, no prospective studies into TART and intensified glucocorticoid treatment have been published so far. Few cases are known in which intensified glucocorticoid treatment has led to reduction of tumor size and improved testicular function in some (5, 59, 75-79) or none (50, 56, 61) of the reported patients. Moreover, glucocorticoid treatment can lead to serious side effects, such as hypertension, striae, and weight gain (78). Especially in adolescents intensifying glucocorticoid treatment is challenging as supraphysiological dosages can impair final height especially in hydrocortisone dosages above 17 mg/m^2/day (97). Glucocorticoids may not lead to shrinkage of the tumor when progressive fibrosis and irreversible damage of the surrounding testes tissue of the tumor are present (14).

One male CAH patient with TART was successfully treated with the adrenal steroid synthesis inhibitor mitotane to restore fertility. However, mitotane causes irreversible chemical adrenalectomy via an unknown mechanism (98) and is therefore only recommended as a last attempt to treat the infertility. Human chorionic gonadotropin combined with follicle-stimulating hormone was administered to one CAH patient with TART and hypogonadotropic hypogonadism, resulting in restored testicular

testosterone production and consequently fertility. Notably, treatment had to be performed for as long as 21 months (99). In addition, successful testis-sparing surgery in small cohorts of CAH patients with TART has been described in two studies, however without long-term follow up and evaluation of gonadal function (56, 100). We described eight adult male CAH patients with longstanding TART in whom testis-sparing surgery was performed but without significant improvement of gonadal function after surgery (58). Testicular biopsies confirmed that these patients already had irreversible damage of the surrounding testes tissue (14). Surgery seems therefore only indicated when severe pain complaints are present, as it did not restore fertility.

To summarize, CAH patients should be informed about the risks of the development of TART and consequently the risk of infertility. As there is no suitable therapy yet to prevent the formation of TART or to treat TART, we advise to monitor CAH patients regularly (yearly from eight years old and once every two or three years from adulthood on (101)) with ultrasound and offer patients with TART semen cryopreservation. Further research should focus on possible druggable targets to prevent the formation of TART. A possible candidate might be ACTH inhibitors, as increased ACTH concentrations seem to play an important role in the development of TART.

TART in other forms of CAH

TART has also been described in CAH patients with other enzymatic defects than 21-hydroxylase deficiency. Seven studies reported on 11-hydroxylase deficient patients, of which in total 42% (14/33) had TART (13, 21, 23, 26, 30, 34, 44). Incidentally, TART is reported in CAH patients with HSD3B2 mutations (38, 102, 103). Patients with 11-hydroxylase deficiency or HSD3B2 mutations also suffer from deficient cortisol concentrations and consequently increased ACTH concentrations. It is therefore likely that also in these patients, elevated ACTH contributes to the development of TART.

Future recommendations

TART is a typical feature in male CAH patients and is the most important cause of infertility in these patients. As CAH is a rare condition it is difficult to include high patient numbers in a clinical study and thus multicenter multinational investigations (which could be based on an international registry of CAH patients with TART that has to be established) are indicated to empower studies to investigate associations with

poor hormonal control, tumor size, gonadal function, fertility, etcetera and to eventually test new treatment options. We proposed that ACTH is the most important stimulating factor in the development of TART and possible treatment strategies might be based on lowering ACTH concentrations. If other stimulating factors, such as angiotensin II, are confirmed therapies on lowering these concentrations might also be indicated. In the latest years, next generation sequencing has taken off. TART research would benefit from performing transcriptome sequencing as it provides extensive data on specific gene expression, thereby enable to elucidate the particular characteristics of TART, possible drug targets, and biomarkers to distinguish TART from Leydig cell tumors. Unfortunately, no relevant cell line or animal model is available to study the development of TART or possible treatment options. More fundamental research is therefore needed to establish a TART model.

Conclusions

TARTs are a common complication in male CAH patients with an overall prevalence of 40% in classic CAH patients. The prevalence is especially high in adults (up to 89%), but it also occurs in children with an increase in prevalence during puberty. TART prevalence seems to be associated with the severity of CAH. TART tissue has adrenal as well as testicular characteristics and is able to produce steroids. As currently no definite therapy is available to treat TART, future research should focus on possible drug targets. Lowering ACTH might prevent the formation of TART as ACTH seems to be involved in the development of TART. We urge all clinicians to advise male CAH patients on the risk of developing TART and its consequences. Furthermore, sperm cryopreservation could be offered to TART patients as there is a high risk on infertility.

Search strategy

Articles were selected after searching PubMed and EMBASE for articles published up to February 2018 using the search terms "congenital adrenal hyperplasia", "adrenogenital syndrome" in combination with "adrenal rest tumo(u)r", "adrenal rest(s)", "adrenal cortical rest tumo(u)r", "TART", "testicular masses / nodules / tumo(u)r / neoplasm", "testicular adrenal tissue", "health status", "long term outcome". From the retrieved set of papers, duplicates and triplicates were manually deleted from Endnote.

Based on title/abstract, primary screening was performed by a single review author (ME), deleting articles that clearly did not involve TART. Secondary screening involved

review of full-text articles in the English language. Inclusion criteria were: original study (excluding case reports), outcome measures should include TART, and regarding prevalence numbers the study should be published in the last 10 years (2009 - 2018). This procedure resulted in 64 studies that focused on TART. Data on prevalence, characteristics, origin and treatment options were extracted. Prevalence data on TART detected by ultrasound (US) was found in 31/64 studies and further analyzed by calculating 95% confidence intervals using the exact binominal confidence interval method. Reference lists of included studies were scanned for relevant studies, especially for information on the origin and treatment of TART, as no original studies were performed. Therefore, for these categories also case reports were included.

Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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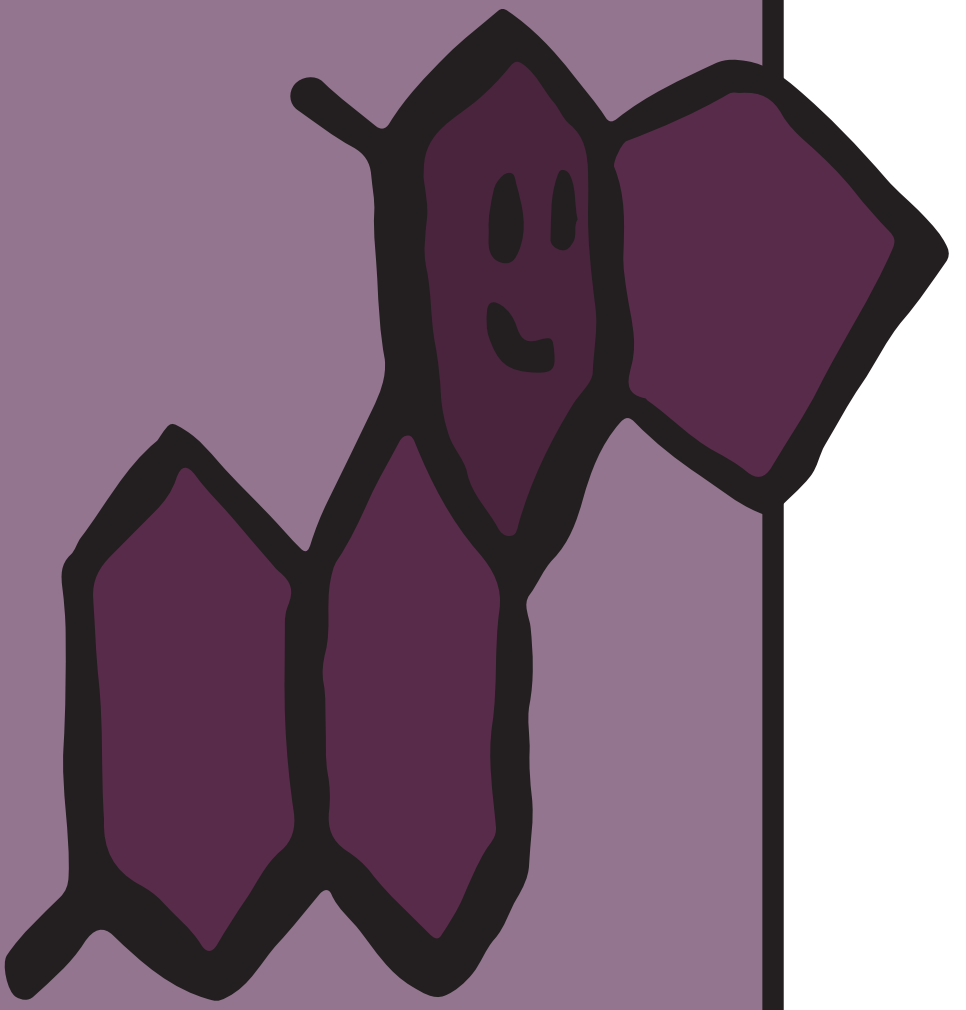

Chapter 5

GATA transcription factors in testicular adrenal rest tumors

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Abstract

Testicular adrenal rest tumors (TARTs) are benign adrenal-like testicular tumors that frequently occur in male patients with congenital adrenal hyperplasia. Recently, GATA transcription factors have been linked to the development of TARTs in mice. The aim of our study was to determine GATA expression in human TARTs and other steroidogenic tissues. We determined GATA expression in TARTs (n=16), Leydig cell tumors (LCTs; n=7), adrenal (fetal (n=6) + adult (n=10)), and testis (fetal (n=13) + adult (n=8)). We found testis-like *GATA4*, and adrenal-like *GATA3* and *GATA6* gene expressions by qPCR in human TARTs, indicating mixed testicular and adrenal characteristics of TARTs. Currently, no marker is available to discriminate TARTs from LCTs, leading to misdiagnosis and incorrect treatment. *GATA3* and *GATA6* mRNAs exhibited excellent discriminative power (area under the curve of 0.908 and 0.816, respectively), while immunohistochemistry did not. GATA genes contain several CREB-binding sites and incubation with 0.1 mM dibutyryl cAMP for 4 hours stimulated *GATA3*, *GATA4,* and *GATA6* expressions in a human fetal testis cell line (hs181.tes). Incubation of adrenocortical cells (H295RA) with adrenocorticotropic hormone (ACTH), however, did not induce GATA expression *in vitro*. Although ACTH did not dysregulate *GATA* expression in the only human ACTH-sensitive *in vitro* model available, our results do suggest that aberrant expression of GATA transcription factors in human TARTs might be involved in TART formation.

Introduction

Congenital adrenal hyperplasia (CAH) is a genetic disorder in which adrenocortical steroid synthesis is impaired due to a deficiency in particular steroidogenic enzymes, most often steroid 21-hydroxylase (CYP21A2). A wide range of the male CAH patients from 12.5% up to 94% are reported to develop testicular adrenal rest tumors (TARTs), which are an important cause of infertility (1, 2). TARTs are benign tumors with steroidogenic characteristics, located near the mediastinum testis (1, 3). Until now, the etiology and origin of TARTs have remained uncertain. TARTs were originally thought to arise from adrenal rest cells, based on the presence of adrenal characteristics, such as expression of adrenal enzymes and receptors (4, 5). However, recently we also described testicular characteristics of TARTs (6). This has shifted the hypothesis toward a more pluripotent steroidogenic cell type as the origin of TARTs (6), possibly from cells originating in the urogenital ridge or adrenogonadal primordium.

Besides exhibiting both adrenal and testicular characteristics, TARTs also share morphological similarities with steroid-producing testicular Leydig cells. As a consequence, it is difficult to discriminate TARTs from Leydig cell tumors (LCTs). Both TARTs and LCTs are rare tumors (7). Although rare entities, several cases of LCTs in CAH patients have been described (8-13). Discrimination between LCTs and TARTs is important as these require different treatment strategies. TARTs are detected using ultrasound or MRI investigation. Currently, TARTs will only be surgically removed from the testis when pain complaints are present (3), while LCTs will be surgically removed using a testis-sparing procedure or total orchiectomy (7). No single marker is available yet to accurately discriminate TARTs from LCTs, increasing the chance of misdiagnosis and consequently incorrect treatment, of which at least 2 cases have been reported in literature (14, 15).

GATA transcription factors are involved in development (by regulating cell fate specification) and differentiation in all eukaryotic organisms. These factors are able to bind to a consensus DNA element, WGATAR, known as the GATA motif (16, 17). Historically, GATA transcription factors are divided into two families: GATA1, GATA2, and GATA3 are classified as hematopoietic factors, while GATA4, GATA5, and GATA6 are classified as endodermal factors. Their expression is also described in almost all fetal and adult tissues, and they are involved in adrenogonadal development. Three GATA factors (GATA 1, 4, 6) are expressed in the somatic cell population of the testis, while GATA3 is expressed in the adrenal medulla (reviewed in Viger *et al.* (18)).

A possible relation between the expression of GATA transcription factors during adrenogonadal development and TART development was proposed in commentaries in

the study of Padua *et al.* (19) by Heikinheimo *et al.* (20) and Pihlajoki (21). Padua *et al.* (19) developed a mouse model lacking both GATA4 and GATA6 expressions in steroidogenic cells. These mice suffer from adrenal aplasia, and female mice die within days after birth. However, male mice survive because of corticoid production by adrenal-like cells in the testes, which Heikinheimo (20) and Pihlajoki (21) proposed might be similar to TART cells. Interestingly, GATA genes contain cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) sites, and cAMP induces expressions of GATA4 and GATA6 in gonadal cell lines (22-24). Levels of ACTH, the receptor of which signals via cAMP, are raised in CAH patients, due to lack of negative feedback on the hypothalamus-pituitary-adrenal axis, caused by low or absent cortisol levels due to the adrenal enzyme deficiency. Furthermore, ACTH levels are associated with the occurrence of TARTs (25-27). Therefore, we hypothesized that dysregulation of GATA transcription factors by increased ACTH levels *in utero* might be involved in the etiology of TARTs.

The aim of our study was therefore to determine the expression of GATA transcription factors in TARTs and other steroidogenic tissues. We determined their discriminative potential to discern TARTs from LCTs and studied the role of cAMP and ACTH in the etiology of TARTs *in vitro*.

Methods

GATA expression analysis in human material

Tissues and patients

Sixteen TART samples from 8 adult CAH patients (tumor left and right testis) were previously collected as described by Claahsen *et al.* (3, 5) (informed consent was obtained). Paraffin-embedded material for immunohistochemistry was available for all tumors, while frozen material for RNA isolation was available for 12 samples. Additionally, 2 frozen histologically proven TART samples from one anonymous CAH patient were obtained. Frozen material of normal testis (*n*=8), normal adrenal (*n*=10) and benign LCT (*n*=7) was obtained. Furthermore, paraffin-embedded material was available for 4 benign LCTs and 3 metastases of malignant LCTs. These coded (identifiable anonymous) testis tissues, adrenal tissues, benign LCTs, metastases of malignant LCTs and TART samples were obtained from the Pathology and Urology departments and used in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/codes-conduct; research approved by institutional review board: CMO Radboudumc #2016-2977 and

CMO-nr 2004/007). To study the etiology of TARTs, we also included fetal adrenal and testis tissues. Six human fetal adrenals (first and second trimesters) and cDNA from 13 fetal testis tissues (second trimester) were obtained from the MRC Centre for Reproductive Health, University of Edinburgh. Tissues were obtained following elective termination of pregnancy and anonymized. Women gave informed consent in accordance with national guidelines (28) and ethical approval was obtained from the Lothian Research Ethics Committee.

RNA isolation

Frozen tissue sections (at least 10×20 µm) or cultured cells were used for RNA isolation (Total RNA Purification kit, Norgen, Thorold, Canada) according to manufacturer's instructions. Samples were treated with DNase (RNase-free DNase set, Qiagen). RNA concentrations and purity were determined using a NanoDrop 2000 Spectrophotometer.

Reverse transcription and qPCR

0.1 µg (fetal testis), 0.2 µg (first trimester adrenal) or 0.5 µg (second trimester adrenal and adult samples) of total RNA was used for cDNA synthesis using Superscript II reverse transcriptase (Thermo Fisher Scientific), performed according to the manufacturer's protocol with a 2720 Thermal cycler (Applied Biosystems) in a final volume of 20 µL. Gene-specific primers of GATA1, GATA3, GATA4, and GATA6 were self-designed (Supplementary Method and Supplementary Table 5.1). For qPCR, the cDNA of adult samples was diluted 5 times, while the cDNA of fetal samples was diluted 20 times, and 2 µL was added to 7.5 µL IQSYBR Green Supermix (Bio-Rad Laboratories), in a total amount of 15 µL on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). As fetal testis cDNA was not DNase-treated, a non-RT control was used to determine genomic DNA contamination.

Immunohistochemistry

GATA3 immunostaining was performed using a standardized protocol optimized for the localization of GATA3 in urothelial carcinoma (antibody L50-823, 1:50 dilution, Biocare Medical/ Klinipath, Duiven, The Netherlands). Kidney sections were used as positive control. GATA6 immunostaining (sc-9055; 1:200 dilution, SantaCruz Biotechnology) was performed manually including negative control sections with only primary antibody diluent. All sections were visualized with VisionTek (Sakura, Tokyo, Japan).

Regulation of GATA transcription factors

Cell culture

The hs181.tes cell line was obtained from American Type Culture Collection (ATCC CRL-7131), while the H295RA cell line was obtained from the University of Michigan (29). Cells were grown as a monolayer culture, although the H295RA cells tend to grow in clumps. Media for hs181.tes cells consisted of DMEM with 4.5 g/L glucose with L-glutamine (Lonza; Leusden, Netherlands), whilst DMEM/F12 (Lonza) was used for H295RA cells. Both media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific) and 1% antibiotics (penicillin-streptomycin 10 000 U/mL; Gibco). Cells were cultured at 37°C in a humidified 95% air/5% CO_2 atmosphere. Medium was changed 2-3 times a week and hs181.tes cells were passaged when confluent using 0.25% trypsin (BD Diagnostic Systems, Breda, The Netherlands), while for H295RA cells 0.05% trypsin-EDTA (Gibco) was used.

Dibutyryl cAMP and ACTH studies

Hs181.tes and H295RA cells were washed, harvested and plated (1:6 dilution) into a 6-well plate (Costar, Corning Life Sciences). After 24 hours, cells were starved overnight using serum-free medium (hs181.tes) or low-serum experimental medium (1% FBS; H295RA). RNA was isolated after cells were treated with 0.1 mM dibutyryl cAMP (Sigma) for either 30 minutes, 4 hours or not treated at all. ACTH incubation experiments were only performed in the H295RA cells, as hs181.tes cells are insensitive to ACTH. RNA was isolated after cells were not treated or treated with 2 or 10 nM ACTH (Synacthen, Radboudumc Pharmacy, Nijmegen, The Netherlands) for either 30 minutes, 4 hours or 24 hours.

Data analysis

Gene expression

mRNA expression of all genes was calculated using the delta Ct method ($2^{-\Delta Ct}$). All values were normalized to the corresponding *HPRT* value (30). Data were transferred to GraphPad Prism 5 and IBM SPSS 22.0 (SPSS Inc.) for further analyses. Differences between different tissues and conditions were tested for statistical significance with non-parametric tests. To determine the diagnostic properties of GATA in discriminating TARTs from LCTs, Mann-Whitney U was performed and following Receiver Operating Characteristic (ROC) analyses were performed. The area under the curve (AUC) represents the probability that the outcome correctly classifies the tissue as TART or benign LCT (range 0.5 (no accuracy) to 1 (prefect accuracy)). To determine the role of GATA in the etiology of TARTs, a comparison between TARTs and testes (fetal and

adult), TARTs and adrenals (fetal and adult) was made. Furthermore, expressions in fetal tissues were compared with each other (adrenal vs. testis), as well as expressions in adult tissues (adrenal vs. testis). Also, expressions in fetal and adult testis, and fetal and adult adrenal tissues were compared. These comparisons were analyzed with the Kruskal–Wallis test followed by Dunn's *post hoc* test. Gene expression analyses within cell culture studies were also compared using the Kruskal–Wallis test followed by Dunn's *post hoc* test. Values of $P \leq 0.05$ (*), or $P \leq 0.01$ (**), or $P \leq 0.001$ (***), were considered statistically significant.

Protein immunohistochemistry

IHC stainings for GATA3 and GATA6 in TARTs and LCTs were visually examined by two independent investigators (MAMvZ, ME). GATA3 and GATA6 were scored based on pseudoquantative histoscore: intensity of staining was recorded as negative, weak, moderate or strong. Furthermore, an estimation of the percentage of positive cells was made. For GATA3, only nuclear staining was scored as GATA3 staining is already validated for urothelial cell carcinoma, indicating only nuclear staining as a positive reaction. In contrast, as GATA6 staining is still experimental, we considered both nuclear and cytoplasmic stainings as positive. For GATA3, nuclear reactivity of the tubular cells of the kidney was used as external positive control for the staining procedure. For GATA6, tissue sections without primary antibody were used as a negative control for the staining procedure. Cytoplasmic staining in GATA6 was corrected for any lipofuscin present by comparing the negative control directly with the GATA6 staining. In the cases of staining heterogeneity, the highest level was reported. Staining scores were compared between the two investigators (MAMvZ, ME) and both agreed on reported (consensus) scores.

Results

Adrenal and testicular expression levels of GATA transcription factors in TARTs

To test whether expression of *GATA1*, *GATA3*, *GATA4,* and *GATA6* is a marker of different tissues or disease states, qPCR was performed (Fig. 5.1). Separate analyses were performed to determine their discriminative potential between TARTs and LCTs (Fig. 5.1 underlined significance) and to determine their role in the etiology of TARTs by comparison with fetal and adult testis and adrenal tissues (Fig. 5.1 non-underlined significance).

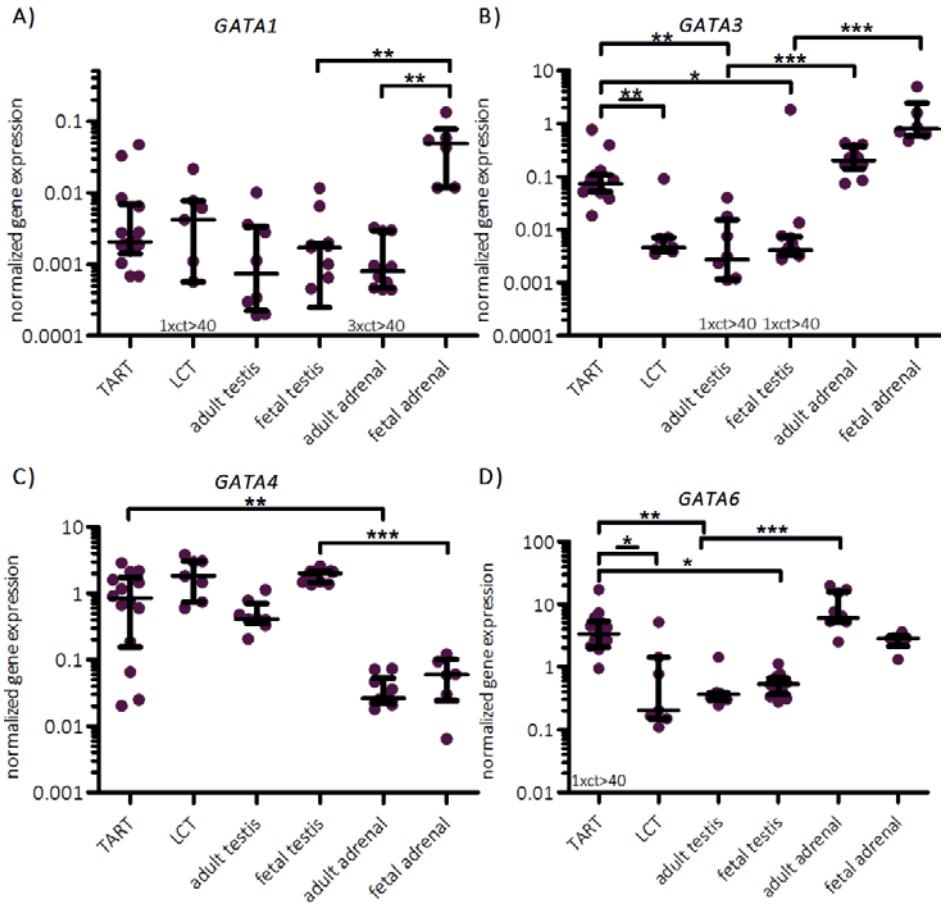

Fig. 5.1: Gene expressions of *GATA1* (A), *GATA3* (B), *GATA4* (C), and *GATA6* (D) in human TARTs, adult testes, fetal testes, adult adrenals, and fetal adrenals. mRNA expression was calculated using the delta Ct method. All values were normalized to corresponding *HPRT* expression. The symbols in the graph represent all samples used and the error bars indicate median and 25th and 75th percentiles. Significance was tested with the Mann-Whitney U (MWU) test or Kruskal–Wallis and Dunn's *post hoc* test comparing selected pairs of variables. Underlined significances are from the biomarker analysis comparing TARTs and LCTs (MWU). Non-underlined significances are from the etiology analysis comparing TART, adult adrenal, adult testis, fetal adrenal, and fetal testis tissues. **P*≤0.05; ***P*≤0.01; ****P*≤0.001; ct>40 means that mRNA was not detected in these samples. Abbreviations: LCTs, Leydig cell tumors; TARTs, testicular adrenal rest tumors.

Expression of *GATA1* was significantly higher in fetal adrenals compared to fetal testes (28.4-fold, *P*≤0.01) and adult adrenals (61.1-fold, *P*≤0.01) (Fig. 5.1A).

GATA3 expression was maximal in fetal adrenals, which was 190-fold higher compared to fetal testis tissues (*P*≤0.001). *GATA3* expression was also significantly higher in adult

adrenals compared to adult testes (73.7-fold, $P \leq 0.001$), and expression in TARTs was significantly higher compared to fetal (17.8-fold, $P \leq 0.05$) and adult (26.9-fold, $P \leq 0.01$) testes (Fig. 5.1B).

Gene expression of *GATA4* was significantly higher (32.4-fold) in TARTs compared to adult adrenals ($P \leq 0.01$). Significantly higher *GATA4* expression (33.5-fold) was found in fetal testis tissues compared to fetal adrenal tissues ($P \leq 0.001$) (Fig. 5.1C).

GATA6 expression levels were 16.6-fold higher in adult adrenals compared to adult testes ($P \leq 0.001$), while *GATA6* expression in TARTs was higher compared to fetal (6.3-fold, $P \leq 0.05$) and adult (9.3-fold, $P \leq 0.05$) testes (Fig. 5.1D).

The results of the gene expression analyses are summarized in Fig. 5.2: *GATA3* and *GATA6* are highly expressed in fetal and adult adrenal tissues, while *GATA4* is highly expressed in fetal and adult testis tissues. TARTs express high levels of *GATA3*, *GATA4* and *GATA6*, indicating adrenal- and testis-like expression patterns of GATA transcription factors.

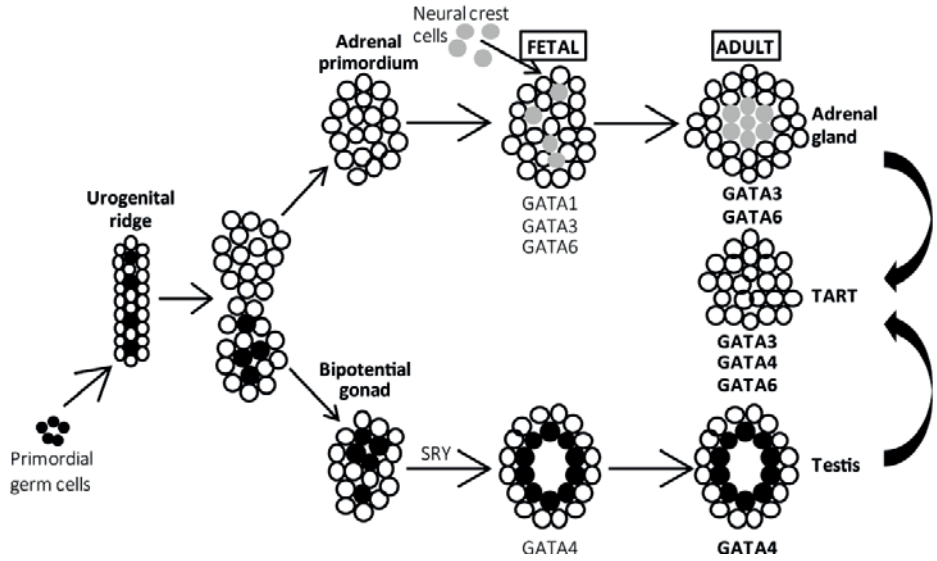

Fig. 5.2: Gene expression of GATA transcription factors during gonadal and adrenal development. The figure summarizes the results of our gene expression analysis in relation to adrenogonadal development. Cells from the adrenal primordium combined with neural crest cells give rise to the fetal adrenal, which will mature into the adult adrenal. The testis develops from the bipotential gonad. In this study, we measured gene expression levels of *GATA1*, *GATA3*, *GATA4,* and *GATA6* in human TART, fetal and adult adrenal, and fetal and adult testis tissues. Of note, we are uncertain of cell-specific expression as we measured expression in total tissue. *GATA3* and *GATA6* were expressed in both fetal and adult adrenals, while *GATA4* was expressed in the fetal as well as the adult testes. *GATA3*, *GATA4,* and *GATA6* gene expressions were all found in TARTs. Abbreviations: TARTs, testicular adrenal rest tumors.

GATA3 and *GATA6* mRNA levels can discriminate TARTs from LCTs, while protein levels cannot

Next, we compared GATA expression and their discriminative potential between TART and LCT tissues, as there is currently no single marker that can distinguish between these two pathologies. *GATA3* gene expression was 15.8-fold higher expressed in TART compared to LCT tissues ($P \leq 0.01$ Fig. 5.1B), and *GATA6* was 16.5-fold higher expressed in TARTs compared to LCTs ($P \leq 0.05$ Fig. 5.1D), while *GATA4* showed no significant difference between TARTs and LCTs (Fig. 5.1C). To determine the discriminative potential in distinguishing TARTs from LCTs based on GATA3 or *GATA6* gene expression, we performed ROC analyses. *GATA3* showed excellent discriminative potential to differentiate TARTs from LCTs with an AUC of 0.908, while *GATA6* showed good discriminative potential with an AUC of 0.816 (Fig. 5.3).

To enhance the clinical applicability of GATAs as TART biomarkers and to determine which cells express GATA, we set out to assess GATA3 and GATA6 expressions using immunohistochemistry on paraffin-embedded formalin-fixed tissues. Protein expression was analyzed in TARTs (n=16), benign LCTs (n=4) and metastases of malignant LCTs (n=3). GATA3 protein expression was undetectable in all TART samples as well as in all LCTs, while the tubular cells of kidney sections (positive control) showed nuclear expression (Fig. 5.4). In TARTs and LCTs, GATA6 protein expression was heterogeneous (Supplementary Fig. 5.1 and Supplementary Fig. 5.2), while expression was absent in negative control samples (Fig. 5.4). Both nuclear and cytoplasmic stainings were observed, with a high variability in intensity and percentage of positive cells. TARTs and benign LCTs show similar intensity and percentage of cells with GATA6 protein expression, while protein expression of GATA6 in metastases of malignant LCTs is almost absent as there are only very few cells with staining (Fig. 5.4).

GATA transcription factors and their possible role in the etiology of TARTs

We hypothesized that prenatal exposure of fetal steroidogenic pluripotent cells to ACTH might induce TARTs via GATA transcription factors. ACTH acts on the ACTH receptor (MC2R), a G-coupled protein, using cAMP as a second messenger. Indeed, one or multiple CREB-binding sites occur inside the gene body or up to 10 kb upstream of the transcription start site of multiple *GATA* genes (http://sabiosciences.com/chipqpcrsearch.php?app=TFBS), suggesting that cAMP could be a GATA expression-inducing second messenger, involved in de- and/or upregulation of GATAs in TARTs.

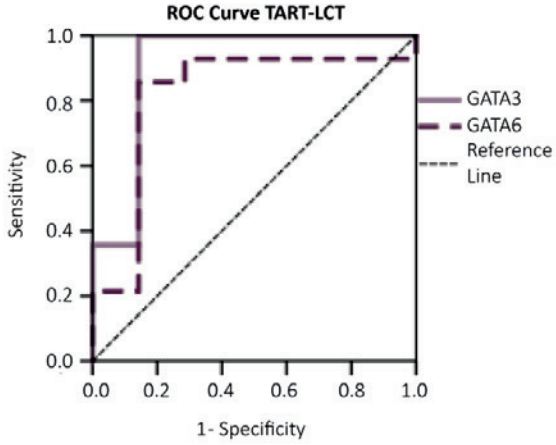

Fig. 5.3: Discriminative potential of *GATA3* and *GATA6* gene expressions in distinguishing TARTs from LCTs. Receiver operating characteristic analyses were performed. An area under the curve of 0.908 was observed for *GATA3*, while this was 0.816 for *GATA6*. Abbreviations: LCTs, Leydig cell tumors; TARTs, testicular adrenal rest tumors.

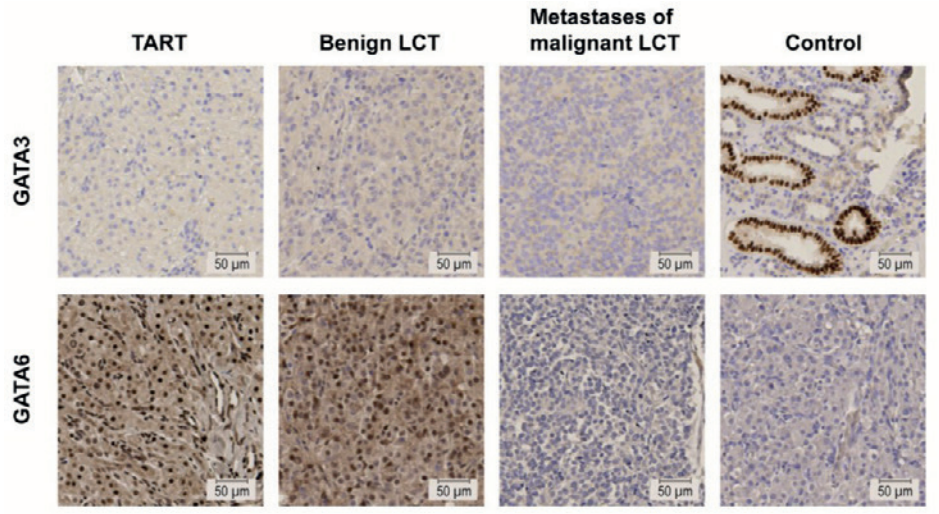

Fig. 5.4: GATA3 and GATA6 protein expressions in human TARTs, benign LCTs, metastases of malignant LCTs and control tissues. GATA3 and GATA6 expressions (positive staining) are indicated by brown nuclei and/or brown cytoplasm. Protein expression of GATA3 is present in the positive control tissue (kidney) but absent in TARTs, benign LCTs and metastases of malignant LCTs. Protein expression of GATA6 is present in the cytoplasm and nuclei of TARTs and benign LCTs, however absent in metastases of malignant LCTs and the negative control (tissue slides without primary antibody). Representative pictures are shown. Scale bar represents a distance of 50 µm. Abbreviations: LCTs, Leydig cell tumors; TARTs, testicular adrenal rest tumors.

Incubation of a fetal testis cell line (hs181.tes) with 0.1 mM of dibutyryl cAMP for 4 hours showed a moderate increase in gene expressions of *GATA3*, *GATA4,* and *GATA6*, although this did not reach statistical significance (Fig. 5.5A). However, there is no *in vitro* model of fetal testis cells expressing MC2R. Therefore, we used an ACTH-sensitive adrenocortical cell line (H295RA). ACTH incubation with 2 nM (results not shown) or 10 nM (Fig. 5.5B) for 30 minutes, 4 hours or 24 hours increased *MC2R* and *CYP11B1* gene expressions (positive controls), indicating that the system is indeed responsive to ACTH. However, no altered gene expressions for *GATA3*, *GATA4,* or *GATA6* were found.

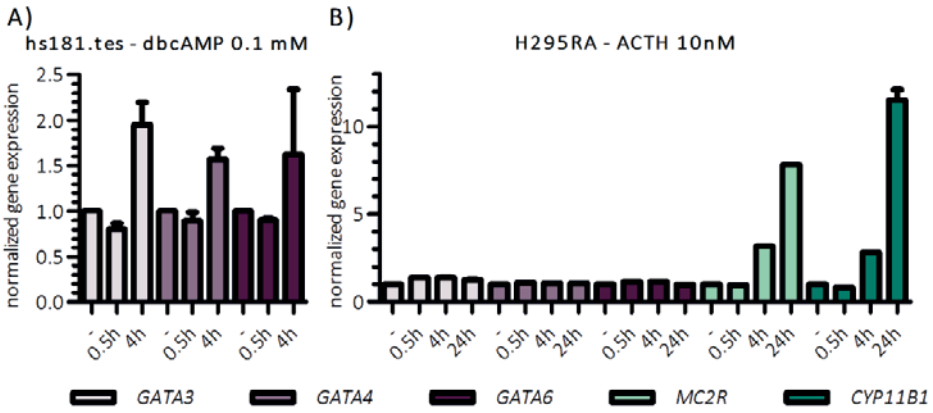

Fig. 5.5: GATA transcription factors and their possible role in the etiology of TARTs. Long-term exposure to elevated levels of ACTH is present in patients with congenital adrenal hyperplasia and this is associated with the development of TARTs. ACTH binds to its receptor (MC2R), using cAMP as a second messenger. CREB-binding sites are present within the gene body or up to 10 kb upstream of the transcription start site in GATA3, GATA4, and GATA6. cAMP could therefore be a GATA expression-inducing second messenger. Source: http://sabiosciences.com/chipqpcrsearch.php?app=TFBS. **A)** Hs181.tes cells were incubated with 0.1 mM dibutyryl cAMP (hs181.tes cells do not express ACTH receptor) for 0, or 30 minutes, or 4 hours. Gene expression was calculated using the delta Ct method and corresponding *HPRT* expression was used to normalize. **B)** H295RA cells were incubated with 10 nM ACTH for 0, or 30 minutes, or 4, or 24 hours. Delta Ct method and corresponding *HPRT* value were used to calculate normalized gene expression.

Discussion

To the best of our knowledge, this is the first description of GATA transcription factors in human TARTs. TARTs expressed both testicular (*GATA4)* and adrenal (*GATA3* and *GATA6*) characteristics, thereby confirming our previous findings of both adrenal and testicular features of TARTs (6). Furthermore, differences in *GATA3* and *GATA6* mRNA

expression levels might be used to discriminate TARTs (high) from LCTs (low expression), indicated by good AUCs (>0.8) in ROC analyses, although at the protein expression level, immunohistochemistry did not discriminate. In addition, as long-term exposure to elevated ACTH levels is linked to occurrence of TART (25-27), we hypothesized that (prenatal) exposure of (primordial) steroidogenic cells in the testes to ACTH might induce TARTs via deregulation of GATA transcription factors. Human fetal testis cells indeed show increased GATA expression after incubation with cAMP. However, adrenocortical cells (the only human ACTH-sensitive model available) did not show increased expression after ACTH incubation.

Although interesting to again find both adrenal- and testis-like characteristics of TARTs, this expression pattern does not correspond to the expression pattern observed in the adrenal-like cells of the GATA4/GATA6 double-knockout mice described earlier (19). The steroidogenic cells in the double-knockout mice should not express GATA4 and GATA6, as the model eliminated expression in all steroidogenic cells. In addition, the adrenal-like cells in these mice lacked *HSD17B3* and *INSL3* expressions, whereas these genes are expressed in human TARTs (19). Therefore, the observed gene expression patterns of adrenal-like cells found in these mice do not resemble the observed gene expression pattern of human TART.

We assessed the potential of GATA transcription factors as differential diagnostic tools to discriminate TARTs from LCTs, which are difficult to distinguish due to their morphological resemblance. Misdiagnosis can have profound consequences for the treatment of a patient with a testicular tumor (8, 11). Therefore, a clinical need for differential diagnostic markers that can differentiate between both pathologies exists. Bilateralism of the tumors (25, 31), presence of Reinke crystals (1, 5, 25, 32-35) and expressions of synaptophysin, Inhibin α, CD56, androgen receptor, DLK1, INSL3, CYP11B1, CYP21A2, and MC2R (34-37) have all been studied as potential markers, but none of these markers individually can reliably discriminate TARTs from LCTs. We found significantly higher gene expressions of *GATA3* and *GATA6* in TARTs compared to LCTs with good discriminative potential. This suggests that measurement of these genes may be used in a diagnostic setting as a discriminative marker between TART and LCT tissues. To improve the usefulness of these markers in the clinic and to determine which cells express GATA, we determined protein expressions of GATA3 and GATA6 on paraffin-embedded tumor samples using standard immunohistochemical techniques. GATA3 protein expression, however, was undetectable in TARTs and LCTs. On the other hand, GATA6 protein expression was heterogeneous both within and between TART, benign LCT and metastases of malignant LCT tissue samples. We observed high variability in the location, intensity of staining and percentage of GATA6-positive cells. We identified very low expression of GATA6 in metastases of malignant LCTs, which

might reflect a change in the status in the primary tumor cells promoting invasion. Nevertheless, GATA6 expression is heterogeneously expressed within and between TARTs and benign LCTs, and it can therefore not be used as a discriminatory biomarker for these pathologies.

We also determined *GATA* expression in human fetal adrenal and testis tissues. *GATA1* was the only GATA gene in which a significant change in expression was identified between fetal and adult adrenal tissues. We found *GATA4* expression in fetal and adult testis tissues, and *GATA6* expression in fetal and adult adrenal. Previous findings are in agreement with our data. Viger *et al.* summarized all known literature on GATA expression in adrenogonadal development, mainly based on mice models (18). However, as we studied human TARTs, we will focus on reported expression in human tissues. Ketola *et al.* found GATA4 and GATA6 mRNAs and protein expressions in human fetal testis tissues, although a decreasing trend with advanced fetal development was observed for GATA6 expression (38). In fetal testis tissues, *GATA4* was the predominantly expressed GATA gene. Jiminez *et al.* found *GATA6* expression in human adult adrenal tissues but no *GATA4* expression (39). This is in agreement with Kiiverii *et al.* who showed *GATA4* and *GATA6* expressions in human fetal adrenal, but only *GATA6* mRNA and protein expression in human adult adrenal (40). In summary, and in correspondence with known literature, we found expression of *GATA4* in fetal and adult testis tissues, and *GATA6* expression in fetal and adult adrenal tissues. In addition, we found *GATA3* expression in fetal and adult adrenal tissues.

We found high relative gene expression levels of *GATA3*, *GATA4* and *GATA6* in TARTs, suggesting that dysregulation of these transcription factors is involved in the etiology of TARTs. GATA genes contain one or several CREB sites and previously cAMP was described to induce GATA4 and GATA6 expressions in the gonadal cell lines MSC-1, mLT and MA-10 (22-24). We therefore stimulated fetal testis cells with cAMP and indeed found moderate increase in expressions of *GATA3*, *4* and *6*. The ACTH receptor (MC2R) signals via cAMP and elevated ACTH levels are present in CAH patients and associated with the development of TARTs (25-27). We hypothesized that dysregulation of GATA expression by ACTH in fetal steroidogenic cells could eventually lead to the formation of TARTs. Regretfully, no human fetal cell lines are available that express functional MC2R. Human cell lines, in general, tend to have low (functional) ACTH receptor expression, although several adrenocortical cell lines, including H295R cells, are known to have at least moderate expression of MC2R (41, 42). ACTH responsiveness in H295R cells was increased by over-expression of MRAP, resulting in the H295RA cell line, the only human ACTH-sensitive cell line available (29). We are aware of the limitation of using a differentiated cell line as a model for the involvement of ACTH in fetal steroidogenic cells. In the present study, we demonstrated that ACTH

does not influence *GATA* expression in H295RA cells. Timing of ACTH exposure might play an important role in GATA dysregulation. Therefore, dysregulation of GATA might still be a key player in the formation of TART and is possibly regulated by ACTH prenatally.

In conclusion, testis-like expression of *GATA4* and adrenal-like expressions of *GATA3* and *GATA6* were observed in TARTs, suggesting that dysregulation of GATA transcription factors in a pluripotent fetal cell is involved in TART formation. Furthermore, gene expression of GATA transcription factors showed good discriminative potential to differentiate TARTs from LCTs, but further studies have to be performed establishing thresholds in less-invasive material, such as blood or urine, to be of applicable use.

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Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

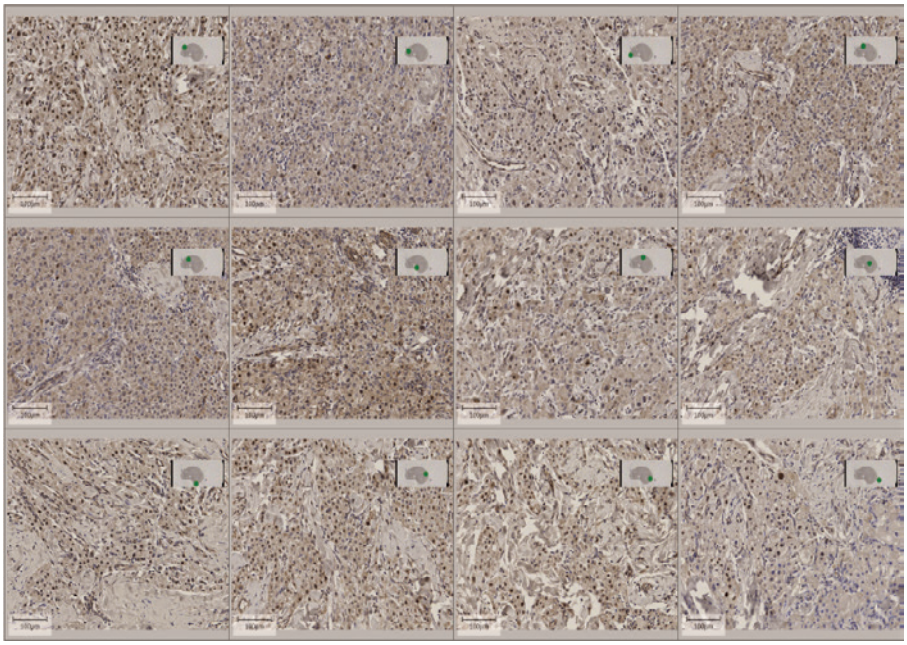
Supplement

Primer design and optimization

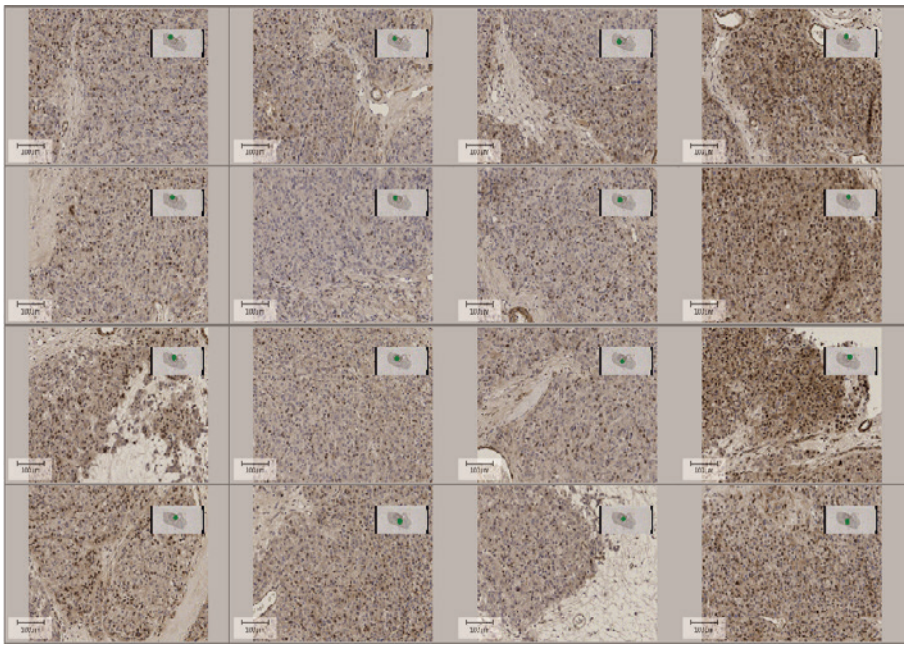
Genes were searched in the NCBI database and imported in PerlPrimer (43). Primers were derived based on an amplicon size of 75-125 base pairs, a length of 17-24 nucleotides, a GC-content of 40-65%, melting temperature of 56-62 °C, an exon-exon junction spanning amplicon and where possible boundary-overlapping primers. Primers were checked for dimers, hairpins and secondary amplicon structures and a set of forward and reverse primers for each gene was ordered (Biolegio, Nijmegen, the Netherlands). The *HPRT* gene was used as normalizing gene (30). Sequences and specifications of all primers are listed in Supplemental Table 1. Efficiency and specificity of the primers were tested using the IQ™SYBR® Green Supermix on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, Netherlands). The PCR products were checked for quality and product size by melting curve analysis and agarose gel electrophoresis.

Supplemental Table 5.1: Primer sequences and specifications.

Gene	Accession Number	Sequence 5' → 3'	Exon boundary overlapping
GATA1 *GATA binding protein 1*	NM_002049.3	FP: AAGAAGCGCCTGATTGTC RP: GCATTTCTCCGCCACAG	yes
GATA3 *GATA binding protein 3*	NM_001002295.1 NM_002051.2	FP: CAGACCACCACAACCACAC RP: TGCCTTCCTTCTTCATAGTCAG	no
GATA4 *GATA binding protein 4*	NM_001308093.1 NM_002052.4 NM_001308094.1	FP: TCTACATGAAGCTCCACGGG RP: TATTCAGGTTCTTGGGCTTCC	no
GATA6 *GATA binding protein 6*	NM_005257.5	FP: GAGGGAATTCAAACCAGGA RP: GTTGGAGTCATGGGAATGG	no
MC2R, melanocortin 2 receptor (ACTH receptor)	NM_000529.2	FP: CAGAGCTGAAGGTGATTGGGA RP: AAGGCGGGGATGTTACTTGG	no
CYP11B1 *11ß-hydroxylase*	NM_000497.3	FP: GGCAGAGGCAGAGATGCTG RP:TCTTGGGTTAGTGTCTCCACCTG	yes
HPRT	NM_000194.2	FP: TATTGTAATGACCAGTCAACAG RP: GGTCCTTTTCACCAGCAAG	yes



Supplementary Fig. 5.1: Heterogeneous GATA6 expression in testicular adrenal rest tumor tissue (TART). 16 random views within a TART. Abbreviations: TART, testicular adrenal rest tumor.



Supplementary Fig. 5.2: Heterogeneous GATA6 expression in Leydig cell tumor tissue (LCT). 16 random views within a benign LCT. Abbreviations: LCT, Leydig cell tumor.

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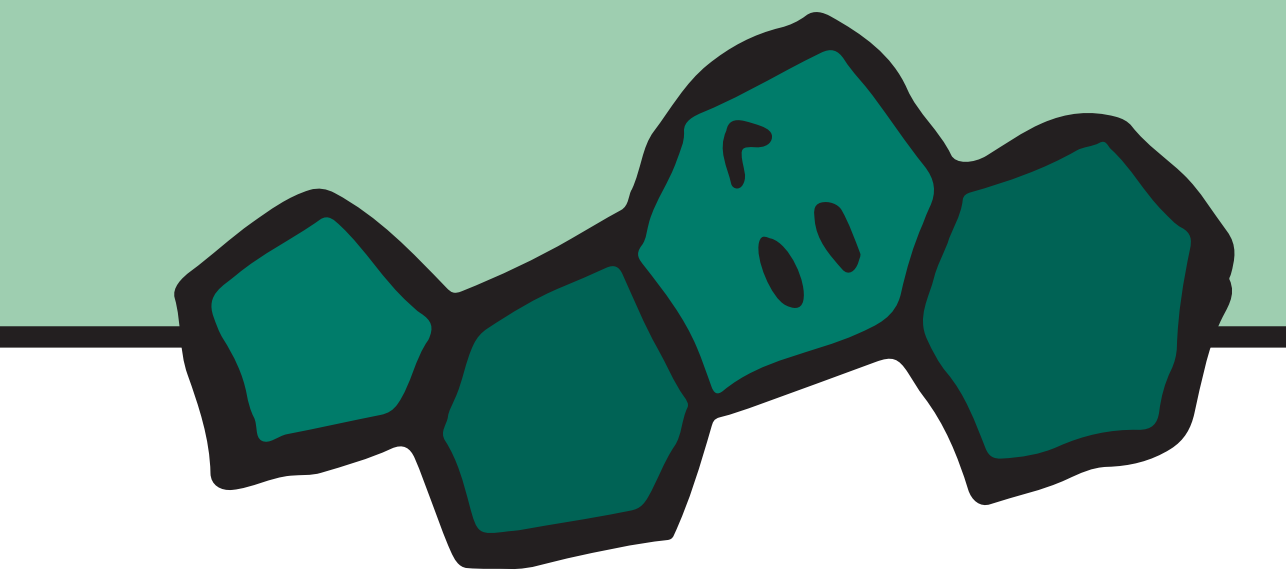

Chapter 6

Bilateral testicular tumors resulting in recurrent Cushing disease after bilateral adrenalectomy

Abstract

Context: Recurrence of hypercortisolism in patients after bilateral adrenalectomy for Cushing disease is extremely rare.

Patient: We present a 27-year-old man who previously underwent bilateral adrenalectomy for Cushing disease with complete clinical resolution. Cushingoid features recurred 12 years later, with bilateral testicular enlargement. Hormonal tests confirmed adrenocorticotropic hormone (ACTH)-dependent Cushing disease. Surgical resection of the testicular tumors led to clinical and biochemical remission.

Design and Results: Gene expression analysis of the tumor tissue by quantitative polymerase chain reaction showed high expression of all key steroidogenic enzymes. Adrenocortical-specific genes were $5.1*10^5$ (*CYP11B1*), $1.8*10^2$ (*CYP11B2*), and $6.3*10^4$ (*MC2R*) times higher than nonsteroidogenic fibroblast control. This correlated with urine steroid metabolome profiling showing 2 fivefold increases in the excretion of the metabolites of 11-deoxycortisol, 21-deoxycortisol, and total glucocorticoids. Leydig-specific genes were $4.3*10^1$ (*LHCGR*) and $9.3*10^0$ (*HSD17B3*) times higher than control, and urinary steroid profiling showed twofold increased excretion of the major androgen metabolites androsterone and etiocholanolone. These distinctly increased steroid metabolites were suppressed by dexamethasone but unresponsive to human chorionic gonadotropin stimulation, supporting the role of ACTH, but not luteinizing hormone, in regulating tumor-specific steroid excess.

Conclusion: We report bilateral testicular tumors occurring in a patient with recurrent Cushing disease 12 years after bilateral adrenalectomy. Using mRNA expression analysis and steroid metabolome profiling, the tumors demonstrated both adrenocortical and gonadal steroidogenic properties, similar to testicular adrenal rest tumors found in patients with congenital adrenal hyperplasia, suggesting the presence of pluripotent cells even in patients without congenital adrenal hyperplasia.

Introduction

Bilateral adrenalectomy is considered a definitive cure for Cushing disease, but some patients may have residual adrenal function from postsurgical remnants or ectopic adrenal tissue (1, 2). Rarely, patients with Nelson syndrome (and high adrenocorticotropic hormone (ACTH) levels) after bilateral adrenalectomy have been reported to develop testicular tumors, with variable cortisol and androgen production (3-5). Some tumors were removed due to symptoms of mass effects, but detailed molecular investigations were not conducted.

In contrast, testicular adrenal rest tumors (TARTs) are seen in up to 94% of male patients with congenital adrenal hyperplasia (CAH), with increasing prevalence during adolescence (6, 7). Elevated ACTH levels, and possibly luteinizing hormone (LH), may play a role in its development (8). We recently demonstrated that TARTs have both adrenocortical and Leydig cell features, suggesting a pluripotent embryonic cell origin (9).

We report a rare case of bilateral testicular tumors resulting in recurrent hypercortisolism in a patient with Cushing disease who had previously undergone bilateral adrenalectomy. We characterized the steroidogenic potential of these tumors by mRNA expression analysis and serum and urinary steroid profiling. Our findings demonstrate that the tumor tissue featured both adrenal and gonadal steroidogenic properties, resembling TART tissue found in patients with CAH.

Case Report

An 11-year-old boy presented with rapid weight gain, rounded facies, and abdominal striae. He was diagnosed with Cushing disease and underwent transsphenoidal removal of a corticotrophin-producing pituitary adenoma. Recurrence occurred within the first postoperative year. After failure of radiotherapy and ketoconazole to control his symptoms, he underwent bilateral adrenalectomy 4 years later (age 15 years), resulting in undetectable serum cortisol after ACTH stimulation (<20 nmol/L). Spontaneous pubertal development ensued. One year later, he developed Nelson syndrome (hyperpigmentation with ACTH 1089 pmol/L) and pituitary apoplexy requiring trepanation and partial extirpation. Subsequently, the pituitary tumor remained stable in size with ACTH levels ranging from 158 to 2921 pmol/L while receiving hydrocortisone 25 mg and fludrocortisone 0.1 mg daily.

At the age of 27 years, 12 years postadrenalectomy, he experienced increased lethargy and weight gain over a period of 6 months. He had a Cushingoid habitus and abdominal striae. His testes were nodular, hard, and enlarged bilaterally. Corticosteroid replacement was stopped, and recurrent ACTH-dependent Cushing disease was confirmed biochemically (Table 6.1). Magnetic resonance imaging showed a small,

Table 6.1: Serum hormones and steroids during dynamic tests and testicular venous sampling after surgery.

		Dynamic testing			Testicular venous sampling				
	Base-line	Base-line [b]	Post-HDDST [c]	Post-hCG [d]	peri-pheral vein	left testicular vein	right testicular vein	Post-surgery [e]	Reference Range
24hour urinary free cortisol	1020								20-135 nmol/24hours
Midnight salivary cortisol	5.6								< 2.2 nmol/L
ACTH	193	293	181	282				1010	2.2-13.2 pmol/L
Renin		27	45	78				54	4.4-85 mU/L
FSH		0.18	0.15	0.19				4.9	1.5-11 U/L
LH		<0.10	<0.10	<0.10				2.8	1.4-8.5 U/L
Inhibin B		63.1	60.9	69.6				<10	150-400 ng/L
Progesterone		3.8	1.0	4.8	2.1	15	69	0.020	<1.3 nmol/L
Corticosterone		14.1	9.2	19.2	27.4	35.5	337	2.6	5.8-56 nmol/L
Aldosterone		<0.09	0.12	0.10	0.43	NA [f]	0.89	<0.03	0.08-0.69 nmol/L
17-hydroxy-pregnenolone		4.17	3.15	3.33	NA [f]	NA [f]	NA [f]	NA	0.9-10.5 nmol/L
17-hydroxy-progesterone		126	38	165	85	880	1780	<0.070	2.0-10.8 nmol/L
11-deoxycortisol		6.1	1.5	6.6	4.0	111	136	<0.17	0.2-4.3 nmol/L
Cortisol		530 [g]	180 [g]	570 [g]	230	2730	2650	<20	190-550 nmol/L
DHEA		2.4	1.3	2.4	1.7	2.1	15.1	<0.2	15-45 nmol/L
DHEAS		3.8	2.8	4.2	2.8	NA [f]	4.1	<0.41	1-7 µmol/L
Androstenedione		58	21	63	36	390	790	<0.060	1.15-4.7 nmol/L
Testosterone		27.0	10.1	27.1	16.9	658	443	<0.1	11.0-45.0 nmol/L
5α-dihydro-testosterone		2.19	1.39	2.39	1.61	NA [f]	5.31	<0.098	1-2.9 nmol/L
Estrone		470	370	430	NA [f]	NA [f]	NA [f]	NA	80-250 pmol/L
Estradiol		80	67	57	81	74	210	25	75-220 pmol/L
11-deoxy-corticosterone		NA	NA	NA	<0.30	0.43	11.7	NA	0.03-1 nmol/L

[a]Testicular vein sampling done during surgery. Testicular veins were cannulated prior to surgical removal. Peripheral sample was taken from a cubital vein during the sampling. [b]Baseline tests were done after exogenous glucocorticoids and mineralocorticoids had been stopped for 1 week. [c]Dexamethasone was administrated orally as 2 mg every 6 hours for 48 hours, with investigations done at 8 AM on the third day, 6 hours after last dose. [d]Daily intramuscular injections of 1500 U hCG were administered subcutaneously on 3 consecutive days at 8 AM. [e]Taken 1 week after surgery, 24 hours after last hydrocortisone dose. [f]Levels not taken due to inadequate serum volume collected. [g]Cortisol levels were measured with immunoassay. Other cortisol levels were measured with liquid chromatography-tandem mass spectrometry. Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; NA, not available.

stable pituitary remnant. Ultrasound and magnetic resonance imaging of the testes revealed a single, large right testicular tumor and multiple left testicular tumors with a small remnant of normal tissue (Fig. 6.1A and 6.1B) Abdominal CT showed a small nodule (<1 cm) in the left adrenal region, suggesting incomplete surgical resection or postsurgical scar tissue. Semen analysis showed azoospermia.

The patient underwent bilateral testicular nodulectomy, with preservation of his left residual normal testicular tissue (Fig. 6.1C-D). One week after surgery, early-morning (8 AM) cortisol was undetectable (<20 nmol/L) (Table 6.1). His symptoms resolved and he was started on corticosteroid and testosterone replacement.

Materials and Methods

A detailed description of the materials and methods is provided in the Supplemental materials and methods. Written informed consent with permission for publication was obtained from the patient and the study was approved by the local ethics committee.

Results

Serum and urine steroid metabolite profiling before and after surgery

Serum and urine were collected at baseline, after high-dose dexamethasone suppression test (HDDST), and after human chorionic gonadotropin (hCG) stimulation. Serum cortisol and its precursors (11-deoxycortisol and 17-hydroxyprogesterone) and androstenedione were elevated at baseline, suppressed by HDDST, and unresponsive to hCG stimulation (Table 6.1). Levels were undetectable after surgery. Testicular vein sampling performed during surgery demonstrated excess steroid production bilaterally (Table 6.1).

Urine steroid metabolite profiling (Fig. 6.1E) showed increased excretion of glucocorticoid metabolites, androgen metabolites (etiocholanolone), and precursor steroid metabolites from all three steroidogenic pathways. The metabolites of 11-deoxycortisol and 21-deoxycortisol were increased up to fivefold, and 17-hydroxyprogesterone metabolites were increased >10-fold of the upper reference range. Dehydroepiandrosterone, which is reflective of the classic androgen synthesis pathway, was low. In contrast, there was highly increased excretion of the alternative androgen pathway intermediates 3α,5α-17-hydroxyprogesterone (485 µg/24hours;

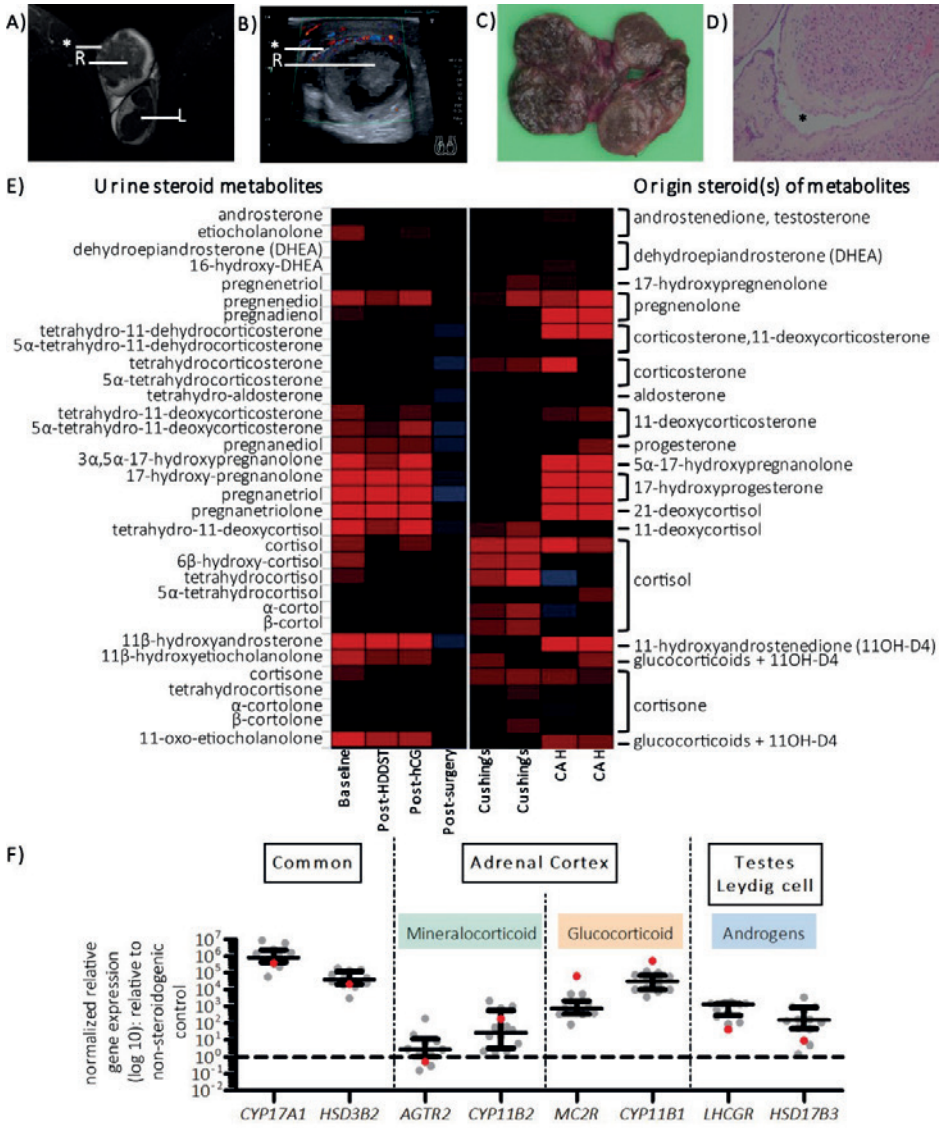

Fig. 6.1: A) MRI Axial T2 turbo inversion recovery magnitude (TIRM) image showing the irregular tumor in the right testis (R), surrounded by a rim of normal testicular tissue (*) and the tumor in the left testis (L). **B**) Sagittal ultrasound image showing the irregular mass in the right testis (R), surrounded by a rim of normal testicular tissue (*).**C)** Macroscopically, both right and left tumors were nodular brown homogeneous tumors, partly encapsulated, measuring up to 5 respectively 6 cm. **D)** Microscopically, the tumors were located in and near the rete testis (*). They had a multinodular aspect consisting of sheets or confluent cords of large polygonal cells with abundant eosinophilic cytoplasm, which infiltrated in between seminiferous tubules, into the rete testis and sporadically intravascular. No other criteria for malignancy (mitosis, necrosis) were met. Incomplete spermatogenesis with maturation arrest at the primary spermatocytic level was seen in the surrounding seminiferous tubules. **E)** Heat map depicting results of 24 hour urine steroid metabolite profiling at baseline, after high-dose dexamethasone suppression test, after hCG stimulation, and after

surgical removal of testicular tumors. Red indicates excretion amounts higher than the adult male reference range, and blue indicates decreased levels. For comparison, 24 hour urine steroid profiling from two adult male patients with ACTH-dependent Cushing's syndrome and two adolescent patients (male on the left, female on the right) with non-classic 21-hydroxylase deficiency are also displayed. **F)** Gene expression profile of 8 markers measured in testicular adrenal rest tumors of congenital adrenal hyperplasia patients (9) (black) and the right testis tumor of the patient (red). The genes were subdivided into common genes for both adrenal cortex and Leydig cells of the testis, adrenocortical specific genes (including mineralocorticoids and glucocorticoids), and Leydig cell specific genes. Symbols and error bars in the graph represent median and 25th and 75th percentile of all normalized relative expression values. Abbreviations: CAH, congenital adrenal hyperplasia; hCG, human chorionic gonadotropin; HDDST, high-dose dexamethasone suppression test.

control group median 26 (range 5 to 118) µg/24hours) and 11-hydroxy-androsterone (15 766 µg/24hours; control group median 588 (range 181 to 1290) µg/24hours).

By comparison, urine steroid metabolite profiling of two newly diagnosed and hence untreated adolescent patients with 21-hydroxylase deficiency demonstrated a similar pattern of increased steroid metabolites, in particular those derived from progesterone, 17-hydroxyprogesterone, 21-deoxycortisol, and the alternative pathway metabolites 3α,5α-17-hydroxyprogesterone and 11β-hydroxy-androsterone. Conversely, these changes were not observed in two adult patients with Cushing disease, who predominantly excreted increased amounts of corticosterone, 11-deoxycortisol, and, in particular, cortisol metabolites (Fig. 6.1E). Thus, the steroid excretion pattern observed in our patient resembled a combination of CAH and Cushing disease.

Gene expression analysis of right testicular tumor tissue

Using quantitative polymerase chain reaction (Fig. 6.1F), the expression of adrenal cortex-specific genes determined to be $5.1*10^5$ (*CYP11B1*), $1.8*10^2$ (*CYP11B2*), and $6.3*10^4$ (*MC2R*) times higher in the patient's tumor tissue compared with the non-steroidogenic control (fibroblast), whereas the expression for *AGTR2* was 2 times lower. Genes common to both adrenal and testis tissue were $3.8*10^5$ (*CYP17A1*) and $2.1*10^4$ (*HSD3B2*) times higher, and Leydig cell genes were $4.3*10^1$ (LHCGR) and 9.3 (*HSD17B3*) times higher compared with nonsteroidogenic control. Compared with TART samples from patients with CAH that we had previously similarly analyzed (9), the patient's tumor was above the interquartile range for glucocorticoid-related factors (*CYP11B1*, *MC2R*) and below the interquartile range for androgen-related factors (*LHCGR*, and *HSD17B3*).

Discussion

In this rare case of bilateral testicular tumors causing recurrence of Cushing disease after bilateral adrenalectomy, we were able to demonstrate by gene expression and steroid metabolome studies that the tumor tissue has shared adrenocortical and gonadal steroidogenic properties.

TARTs are historically considered to originate from adrenal rests descending together with the testes during embryonic development (8). However, we recently found evidence that they develop from pluripotent cells within the testes with adrenal and Leydig cell features (9). Our patient's tumor shared morphological and biochemical features with adrenal tissue, Leydig cell tumors, and virilizing adrenocortical tumors, illustrating the difficulty of differentiating adrenocortical cells from Leydig cells. In a previous similar case, Hamwi *et al.* (10) demonstrated via *in vitro* studies that the testicular tumor in a patient with recurrent Cushing disease was able to produce cortisol and cortisone, although it did not produce androgens. We now demonstrate expression of adrenal-specific genes in the tumor tissue and have correlated this with increased glucocorticoid excretion. In addition, androgen excretion from both classic and alternative synthesis pathways (11, 12) was significantly increased, although relative gene expression of Leydig cell-specific genes was only slightly increased. Interestingly, the steroid excretion profile of the TART tumor in this patient resembled a combination of steroid excretion in CAH and Cushing disease with ACTH-mediated stimulation of intact steroidogenesis.

It is postulated that chronically elevated ACTH, LH, and angiotensin II contribute to differentiation of the early cell (even prenatally) and TART development (8, 13, 14). In our patient, the response to dexamethasone and the high *MC2R* expression support the role of ACTH, which was persistently elevated. However, in this case, exposure was only postnatal, suggesting the presence of pluripotent cells within the testes also in postnatal life and adulthood. Conversely, no cases of post bilateral adrenalectomy TART development have been described in adults older than 23 years, suggesting that regression of pluripotent cells ensues shortly after puberty.

There was relatively higher expression of *CYP11B1* and *MC2R,* but lower expression of *HSD17B3* and *LHCGR* compared with TARTs in patients with CAH (9). This suggests that our patient's tumor was more adrenal like, whereas TARTs in patients with CAH are more Leydig cell like, similar to other findings (3, 10). It has also been suggested that a LH surge during puberty is important for TART development and progression in patients with CAH (6). Although our patient had low relative expression of *LHCGR* and no response to hCG, he entered into puberty soon after adrenalectomy, where he was

exposed to high LH levels. Hence, it remains conceivable that early prepubertal LH stimulation of testicular tissue contributed to TART development.

In conclusion, we present a rare case of recurrent Cushing disease after bilateral adrenalectomy from testicular tumors with adrenal and Leydig cell-specific features. Endocrinologists should consider this possibility if symptoms recur after adrenalectomy while patients are on maintenance steroids. Further research is needed to understand the mechanism of development of these testicular tumors.

Disclosure Summary: The authors have nothing to disclose.

Supplemental Materials

Serum Hormonal Assays

Serum androstenedione, cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, and testosterone, were assessed by an in-house SPE-LCMSMS. Samples underwent solid phase extraction (Oasis, HLB) after protein precipitation (ACN, 0.1% HCOOH). Analysis was performed by LCMSMS (Agilent 6490) with use of an Acquity BEH C18 1.7uM 2.1X50 mm column. Aldosterone, corticosterone, DHEA, dihydrotestosterone, 11-deoxycorticosterone and estrone were assessed by in-house RIAs after extraction and chromatography with recovery correction. Inhibin B was measured by ELISA (gen II; Beckman-Coulter, Woerden, the Netherlands). ACTH, DHEAS, estradiol, LH and FSH were measured by ECLIA (E170, Roche). Plasma renin was measured by immunoradiometric assay provided by CIS Bio (Codolet, France). All methods used have been validated for use in routine clinical diagnostics.

Urine steroid metabolite profiling

Measurement of 24 hour urinary steroid metabolite excretion was carried out by a well-established method employing gas chromatography-mass spectrometry (GC/MS) in selected-ion-monitoring mode for quantification of 38 distinct steroid metabolites. Urine samples had been stored at -20°C before analysis, which was carried out within three months of collection. A detailed description of this methodology has been published previously (15). In summary, free and conjugated steroids were extracted from 1 mL urine by solid-phase extraction. Steroid conjugates were enzymatically hydrolyzed, re-extracted, and chemically derivatized to form methyloxime trimethyl silyl ethers. GC/MS was carried out on Agilent 5973 instrument operating in selected-ion-monitoring (SIM) mode to achieve sensitive and specific detection and quantification of 32 selected steroid metabolites. These represented important steroid groups, such as androgen metabolites, glucocorticoid metabolites, mineralocorticoid metabolites, and 3β-hydroxy-Δ5 steroid precursors (16).

Spermatic vein sampling

The patient underwent bilateral testicular tumor enucleation under general anesthesia. Spermatic vein sampling was done prior to enucleation as described before (17). Briefly, via an inguinal incision and after opening of the inguinal canal, the right spermatic cord was exposed. Care was taken not to manipulate the testes to prevent unwanted secretion of hormones into the circulation. The spermatic vein was cannulated and blood samples were collected to measure serum hormones. The same procedure was performed on the left side. Peripheral blood was collected from a

cubital vein to measure the same hormones. All sera were stored at -20°C until measurements.

Tissue processing and qPCR

All removed tumor tissue was investigated macroscopically and microscopically. Shortly after resection, a portion of the right testicular tumor sample was snap-frozen and kept in liquid nitrogen for mRNA analysis. Frozen tissue sections (30x30 µm) were used for RNA isolation (Total RNA Purification Kit: Norgen Biotek Corporation, Thorold, Canada). Tissue sections were homogenized by addition of lysis buffer and pushing the homogenate through a 21 gauge needle using a 1 mL syringe. Further steps of the isolation were performed according to the manufacturer's protocol. RNA concentration and quality were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). In addition, previous isolated RNA of TART samples of patients with CAH (9) and RNA of healthy control fibroblasts was included as a non-steroidogenic control.

Eight genes were selected for the characterization of the tumor tissue, including the genes encoding 5 key steroidogenic enzymes involved in the adrenocortical and testicular steroid synthesis. The steroidogenic pathways were divided in three parts: common to both adrenal cortex and Leydig tissue (*CYP17A1, HSD3B2*), adrenocortical-specific (*CYP11B1, CYP11B2, AGTR2, MC2R*), or Leydig-specific (*HSD17B, LHCGR*), as previously described (9). Gene-specific primers were obtained and tested as previously described (9). 0.5 µg of total RNA in a volume of 20 µL was used for cDNA synthesis using Superscript II (200U/µL, Gibco) random primers (0.25 µg/µg RNA, Promega), and oligo dTs (0.25 µg/µg RNA, Santa Cruz). cDNA synthesis was performed using the following cycle conditions: 10 minutes at 21°C, 45 minutes at 42°C, 15 minutes at 70°C, 10 minutes at 4°C, on a 2720 Thermal cycler (Applied Biosystems). For qPCR, the cDNA samples were diluted 5 times and 5 µL was added to 7.5 µL iQ™ SYBR® Green supermix (Bio-Rad Laboratories), in a total amount of 15 µL on a CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories). The PCR products were checked for quality by melting curve analysis.

Data analysis

For the gene expression analysis, we calculated the mRNA expression of the genes using the delta-delta C_t method ($2^{-\Delta\Delta Ct}$). All expression values of the patient's tumor were normalized to the corresponding HPRT expression value and then relative to a non-steroidogenic control. Results, previously published, of patients with TART are also shown in Fig. 6.1F for comparison (9).

For the urine steroid metabolites, the patient's results are presented with the median and interquartile ranges of a normal healthy control population, and we used the Cleveland algorithm implemented in SigmaPlot (Systat Software Inc., Chicago, IL).

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Chapter 7

Gonadal function in adult male patients with congenital adrenal hyperplasia

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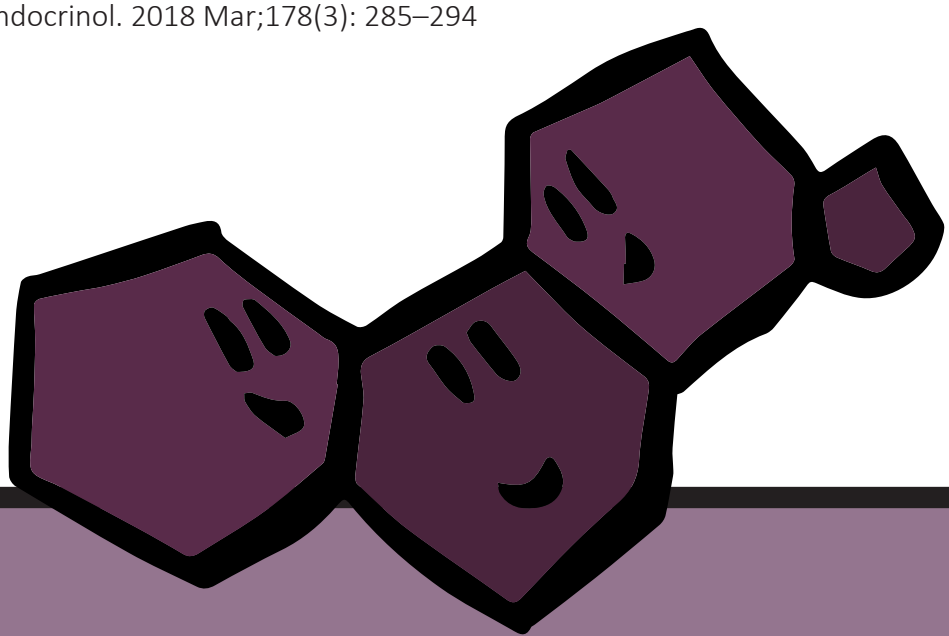

Abstract

Context: Current knowledge on gonadal function in congenital adrenal hyperplasia (CAH) is mostly limited to single-center/country studies enrolling small patient numbers. Overall data indicate that gonadal function can be compromised in men with CAH.

Objective: To determine gonadal function in men with CAH within the European 'dsd-LIFE' cohort.

Design: Cross-sectional clinical outcome study, including retrospective data from medical records.

Methods: Fourteen academic hospitals included 121 men with CAH aged 16-68 years. Main outcome measures were serum hormone concentrations, semen parameters and imaging data of the testes.

Results: At the time of assessment, 14/69 patients had a serum testosterone concentration below the reference range; 7 of those were hypogonadotropic, 6 normogonadotropic and 1 hypergonadotropic. In contrast, among the patients with normal serum testosterone (55/69), 4 were hypogonadotropic, 44 normogonadotropic and 7 hypergonadotropic. The association of decreased testosterone with reduced gonadotropin concentrations (odds ratio (OR)=12.8 (2.9-57.3)) was weaker than the association between serum androstenedione/testosterone ratio ≥1 and reduced gonadotropin concentrations (OR=39.3 (2.1-732.4)). Evaluation of sperm quality revealed decreased sperm concentrations (15/39), motility (13/37) and abnormal morphology (4/28). Testicular adrenal rest tumor (TART)s were present in 39/80 patients, with a higher prevalence in patients with the most severe genotype (14/18) and in patients with increased current 17-hydroxyprogesterone (20/35) or androstenedione (12/18) serum concentrations. Forty-three children were fathered by 26/113 patients.

Conclusions: Men with CAH have a high risk of developing hypothalamic-pituitary-gonadal disturbances and spermatogenic abnormalities. Regular assessment of endocrine gonadal function and imaging for TART development are recommended, in addition to measures for fertility protection.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder resulting in impaired adrenocortical steroid synthesis by several enzyme deficiencies. The most common form (>95%) is 21-hydroxylase deficiency (21OHD) with an incidence of 1:15 000, leading to glucocorticoid and often also mineralocorticoid deficiency in combination with androgen excess (1, 2).

Reported fertility and fecundity in men with CAH on routine steroid replacement therapy range from normal to severely impaired. Fertility can be compromised due to primary (hypergonadotropic) hypogonadism or central (hypogonadotropic) hypogonadism (3-11). In addition, reduced fertility and fecundity rates in CAH can be caused by psychosexual factors (4). Central or secondary hypogonadism is defined as decreased testosterone concentrations in combination with either low or low-normal LH or FSH concentrations. In men with CAH, secondary hypogonadism is most likely caused by the suppressive effect of elevated adrenal androgens (that are aromatized to estrogens) on the hypothalamus-pituitary-gonadal (HPG) axis (6). Differentiation between gonadal and adrenal testosterone is difficult, complicating the diagnosis of hypogonadism in men with CAH. One of the commonest complications in men with CAH is the presence of testicular adrenal rest tumor (TART)s, which can cause disturbances of gonadal function, including mechanical obstruction of the seminiferous tubules. The reported prevalence of TARTs ranges between 12.5% and 94% in the populations studied (4-10, 12-22).

Until now, the data on fertility outcome in men with CAH are scarce (3-11) and often derived from studies with patients from a single center or country. Our aim was to study gonadal function in a large European multicenter cohort of male patients with CAH by evaluating hormone concentrations, semen parameters and TART frequency.

Subjects and methods

Subjects

dsd-LIFE is a cross-sectional clinical outcome study of individuals with disorders/differences of sex development (DSD). Fourteen study centers in 6 European countries (France (n = 4), Germany (n = 4), United Kingdom (n = 1), Poland (n = 2), Sweden (n = 1) and the Netherlands (n = 2)) included former and current patients as participants from February 2014 to September 2015. In addition to DSD participants, 121 male participants with CAH (46XY karyotype) aged 16-68 years were recruited as

they may face similar clinical challenges as DSD patients, including sex hormone imbalances and fertility problems, although male patients with CAH do not fit into the classification of DSD. Written informed consent was obtained from all participants and/or their parents, with assent of minors. Ethical approvals were obtained as appropriate for each country. The theoretical and methodological framework of the dsd-LIFE study have been published in detail (23). Patients were investigated in their local treatment center. Cross-sectional data were obtained for serum hormone concentrations, semen parameters and testicular imaging. The genotype of patients with 21OHD was classified into genotype groups null, A, B, and C (24). General patient characteristics and clinical parameters included: country of inclusion, age, age at diagnosis, CAH genotype and phenotype, socioeconomic status and obesity, as well as height, weight and BMI throughout the years (at diagnosis, 9 months old, 6 years old, Tanner stage 2, 16 years old and current age). Patients' educational levels were established according to the EU classification. We combined the standardized ES-ISCED (international standard classification of education) scale to low (ES-ISCED I = less than lower secondary and ES-ISCED II = lower secondary); medium (ES-ISCED IIIb = lower tier upper secondary; ES-ISCED IIIA = upper tier upper secondary; ES-ISCED IV = advanced vocational, sub-degree) and high (ES-ISCED V1 = lower tertiary education, BA level; ES-ISCED V2 = higher tertiary education, ≥MA level). Data were collected during medical examination at study inclusion (cross-sectional) and retrieved from medical records (retrospective data).

Hormonal analysis

Blood samples were taken during daytime, but mostly in the morning, before intake of the glucocorticoid medication (23). Total testosterone, sex hormone-binding globulin (SHBG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), inhibin B, Anti-Müllerian hormone (AMH), androstenedione, 17-hydroxyprogesterone concentrations and renin/plasma renin activity were measured in the local hospital laboratory and compared to local references. Values are reported in SI or international units and reported as 'below reference range', 'within reference range', 'above reference range up to twice the upper limit' and 'more than twice the upper limit of the reference range'. To increase the number of patients per category, we combined the latter 2 categories into the category 'above reference range'.

The serum androstenedione/testosterone ratio (AD/T) was calculated and divided into normal (<0.5; interpreted as testosterone mainly of testicular origin), ≥0.5 and <1 (significant fraction of testosterone is of adrenal origin) and ≥1 (testosterone mainly of adrenal origin) as suggested by others (25).

Semen analysis

Semen analysis was performed by the local hospital laboratory and interpreted in accordance with the 2010 World Health Organization criteria (26), including sperm concentration (lower reference limit (LRL): 15×10^6/mL), motility (LRL: 40%), morphology (LRL: 4%), vitality (LRL: 58%) and volume (LRL: 1.5 mL).

Imaging of testes

At the study visit, 68 patients (56.2%) underwent testicular ultrasound. The presence of TART at the age of 16 years was also reported retrospectively (in 30/68 patients with cross-sectional TART data). In addition, retrospective data were available for 12 participants based on ultrasound findings or MRI (*n*=11) and on histological findings (*n*=1).

Paternity

Data about paternity and relationships were collected from the dsd-LIFE questionnaires (23).

Medication and estimation of metabolic control in the past

Patients used different formulations of glucocorticoids, including hydrocortisone, prednisone, prednisolone and dexamethasone. Furthermore, we converted all glucocorticoid preparations to hydrocortisone equivalents, using the following factors for the glucocorticoid equivalent dose: 1 (hydrocortisone), 4 (prednisone or prednisolone), 30 (dexamethasone) and 15 (fludrocortisone) (27). We also calculated mineralocorticoid equivalent dose using the following factors: 1 (hydrocortisone), 0.8 (prednisone or prednisolone), 0 (dexamethasone) and 200 (fludrocortisone) (27). In addition to the serum 17-hydroxyprogesterone concentrations presented in the section hormonal analysis, we also assessed metabolic control by a subjective rating, of the local examining physician at 5 different time points: at diagnosis, at the age of 9 months, at Tanner stage 2, at age 16 years and at study inclusion, using the following scores: ‘poor’, ‘moderate’, ‘good’, ‘excellent’ or ‘unknown’.

Statistical analysis

SPSS Statistics 22 (SPSS) was used for all analyses. Descriptive analyses were performed for all variables. Depending on normality, mean and 95% confidence intervals (95%CI) or median and interquartile ranges (IQR) were calculated. We compared patients with values below or above reference range to patients with normal values (within the reference range). Odds ratios (OR) with 95%CI were calculated if at least 3 cases were

present in both subgroups. If any cell count in the contingency table was zero, OR and 95%CI were calculated manually by using a continuity correction (+0.5 in each cell).

Missing data were evaluated for each variable and the total number of participants in a particular analysis was reported exactly. Analysis of the variables was performed only if the number of participants was ≥25% of the total cohort of male patients with CAH.

Three patients were excluded from part of the analyses as they received testosterone substitution, which directly affects testosterone and gonadotropin concentrations. Two of these patients had data on TART available; these are described in the 'Results' section. Furthermore, we excluded 22 patients with missing genotype information and 2 patients with 11β-hydroxylase deficiency from all comparative analyses.

Results

General characteristics of the male CAH cohort

A total of 121 male patients were included in the CAH cohort in the dsd-LIFE study. General characteristics are shown in Table 7.1. The median age of the study population was 28 years (IQR: 18.5-37.5, range 16-68). Mean height was 170.7 (95%CI: 169.3-172.0) cm and median BMI was 25.6 (IQR: 22.0-29.2) kg/m^2 (data available for 119 patients). Nearly all patients had 21OHD (119/121), and 97 were confirmed by molecular genetic analysis. The remaining 2 patients had 11β-hydroxylase deficiency. Among the 97 patients with genetically confirmed 21OHD, 24.7% were classified as genotype null, 38.1% as genotype A, 34.0% as genotype B and 3.1% as genotype C. Glucocorticoids were used by 116 (95.9%) patients, most commonly hydrocortisone, followed by prednisone or prednisolone and dexamethasone. Fludrocortisone was used by 86 patients (71.1%). The patients' education was intermediate or high in 54.5% and 22.3%, respectively. Furthermore, 54.6% of the patients were in a relationship at the time of study.

We analyzed all variables mentioned in the 'Methods' section, but we only present in detail the data that differed between the analyzed groups (no overlap in the confidence intervals). In the following sections, we will present data regarding hormone concentrations, semen analysis and TART.

Hormone concentrations

Univariate descriptive analyses of hormone concentrations were performed. The proportion of patients with normal, decreased or increased serum testosterone, LH,

FSH, inhibin B, AMH and SHBG concentrations is illustrated in Fig. 7.1A. Hormone concentrations were below the reference range in 19/97 (19.6%: testosterone), 8/43 (18.6%: inhibin B), 12/90 (13.3%: LH), 9/90 (10.0%: FSH) and 1/69 (1.4%: SHBG) of the participants. SHBG concentrations were above the reference range in 14.5% (10/69).

Table 7.1: General characteristic of 121 male patients with congenital adrenal hyperplasia. Continuous variables are displayed as median (IQR) or mean (95%CI), depending on normality of the data. Categorical variables are displayed as number of patients and percentage. Patients with 21-hydroxylase deficiency were classified according to severity of the disease. Genotype was classified in genotype group null (0) to group C (24).

Parameter	N	Level	Cohort results
Age	121		28 (IQR: 18.5-37.5) years
Severity of disease	121	*Genotype*	
		21OHD group 0	24 (19.8%)
		21OHD group A	37 (30.6%)
		21OHD group B	33 (27.3%)
		21OHD group C	3 (2.5%)
		No mutation analysis done	22 (18.2%)
		CYP11B1 mutation	2 (1.7%)
Medication	121	Hydrocortisone*	67 (55.4%)
		Prednisone, Prednisolone or Hydrocortisone & Prednisolone^	32 (26.4%)
		Dexamethasone or Hydrocortisone & Dexamethasone#	17 (14.0%)
		No medication	3 (2.5%)
		Fludrocortisone alone	2 (1.7%)
		Fludrocortisone given in addition to any of the glucocorticoid combinations above	84 (69.4%)
Testosterone substitution	121		3 (2.5%)
Height	119		170.7 (95%CI: 169.3 - 172.0) cm
BMI	119		25.6 (IQR: 22.0 - 29.2) kg/m^2
Education	112	Low	15 (13.4%)
		Intermediate	61 (54.5%)
		High	25 (22.3%)
		other, n/a	11 (9.8%)
Partnership situation	108	In current relationship - yes	59 (54.6%)
		In current relationship - no	49 (45.4%)

*Hydrocortisone retard was used by 2 patients. ^A combination of prednisolone and hydrocortisone was used by 1 patient, 17 patients were on prednisolone, 13 on prednisone, and 1 on prednisone retard. #Dexamethasone was used combined with hydrocortisone in 9 patients, while 8 patients were on dexamethasone. †Fludrocortisone given in addition to any of the glucocorticoid combinations listed. Abbreviations: 21OHD, 21-hydroxylase deficiency; 95%CI, 95% confidence interval; IQR, interquartile range; N, number of patients.

Table 7.2 compares testosterone and gonadotropin concentrations in all patients with data on testosterone, LH and FSH available. Seven patients (50%) with decreased testosterone concentrations had decreased gonadotropins, while 6 (42.9%) had normal LH and FSH concentrations and 1 (7.1%) patient had gonadotropin concentrations above reference range. Normal testosterone concentrations were found in 55/69 (79.7%) patients, 44 (80.0%) of whom had normal gonadotropin concentrations, whereas 7 (12.7%) had increased and 4 (7.3%) had decreased concentrations. Decreased testosterone concentrations were clearly associated with decreased LH and/or decreased FSH concentrations (OR: 12.8, 95%CI: 2.9-57.3).

A serum AD/T ratio was calculated in 49 patients, 22 of whom (44.9%) had an AD/T ratio ≥1. Ten patients (45.5%) with an AD/T ≥1 had decreased gonadotropins, while 11 (50.0%) patients had normal gonadotropins and only 1 (4.5%) patient had increased gonadotropins. Normal AD/T ratios were found in 27/49 (55.1%) patients, 21 of whom had normal gonadotropin concentrations (77.8%), 5 had increased concentrations, but none had decreased gonadotropin concentrations. An AD/T ratio ≥1 was strongly associated with decreased LH and/or decreased FSH concentrations (OR: 39.3, 95%CI: 2.1-732.4).

Table 7.2: Testosterone concentrations, androstenedione/testosterone ratios and gonadotropin concentrations in 83 male patients with congenital adrenal hyperplasia. Testosterone, gonadotropin concentrations and androstenedione/testosterone (AD/T) ratio are presented to identify patients with hypogonadism and with prevalent adrenal-derived hyperandrogenism. The number and percentage of patients in each category are given. Odds ratios with 95% confidence intervals were calculated (testosterone concentrations vs. decreased or normal gonadotropins and testosterone concentrations vs. increased or normal gonadotropins).

	decreased LH and/or decreased FSH, n (%)	normal LH and normal FSH, n (%)	increased LH and/or increased FSH, n (%)	Decreased gonadotropins, OR (95%CI)
decreased T	7 (50%.0)	6 (42.9%)	1 (7.1%)	12.8 (2.9 - 57.3)
normal T	4 (7.3%)	44 (80.0%)	7 (12.7%)	
AD/T>1*	10 (45.5%)	11 (50.0%)	1 (4.5%)	39.3 (2.1 - 732.4)
normal AD/T	0	21 (77.8%)	6 (22.2%)	

*An AD/T ratio >1 suggests that the testosterone is mainly of adrenal origin. Abbreviations: AD/T, androstenedione/testosterone ratio; T, testosterone.

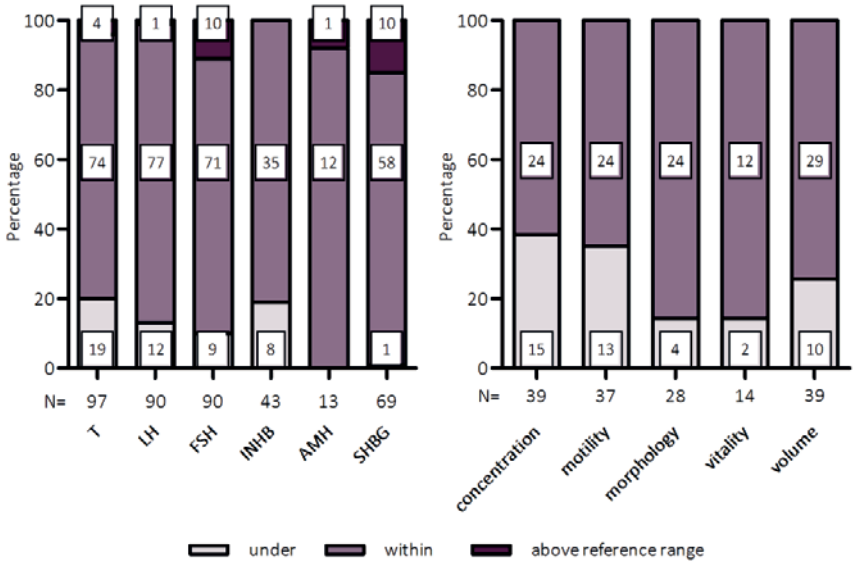

Fig. 7.1: Hormone concentrations (A) and semen quality (B) in male patients with congenital adrenal hyperplasia. Stacked bars represent percentage of patients within a category. Numbers in the bars represent the specific number of patients within a category, while the total number of patients included in this analysis is stated underneath the x-axis. **A)** Hormone concentrations of each patient were measured in the local hospital and compared to the hospitals standard reference ranges. **B)** Semen analysis was performed and scored according to World Health Organization 2010 criteria (26): sperm concentration, motility, morphology, and vitality, and semen volume were assessed. Abbreviations: AMH, anti-Müllerian hormone; INHB, inhibin B; N, number of patients; T, testosterone.

Semen analysis

Semen analysis was performed in approximately one-third of the patients (Fig. 7.1B). Normal values for all known (at least 3 out of 5) semen parameters (normozoospermia) were seen in 11/39 patients in whom semen analysis was performed. Sperm concentration, motility and volume were below the normal ranges in 38.5% (15/39), 35.1% (13/37) and 25.6% (10/39) of the patients, respectively, while morphology and vitality were both impaired in 14.3% (4/28 and 2/14) of the patients. Five of 8 patients (62.5%) with decreased testosterone and gonadotropin concentrations underwent semen analysis, with 4 (80.0%) of them showing abnormal semen parameters (Table 7.3). In only 2/10 patients with decreased testosterone but normal gonadotropin concentrations, semen analysis was performed and both had decreased sperm concentrations (7.0 and $10.0*10^6$/mL). No statistically significant associations were found.

Table 7.3: Semen parameters of 5 male patients with CAH and decreased testosterone and gonadotropin concentrations.

	sperm concentration, LRL: $15x10^6$/mL	sperm motility, LRL: 40%	sperm morphology, LRL: 4%	sperm vitality, LRL 58%	sperm volume, LRL: 1.5mL
p1	$15x10^6$/mL	35%	2%	n.d.	normal
p2	normal	2%	n.d.	58%	1.1mL
p3	$7x10^6$/mL	normal	normal	n.d.	normal
p4	normal	35%	normal	n.d.	1.4mL
p5	normal	normal	normal	n.d.	normal

Eight patients had decreased testosterone and gonadotropin concentrations, of which 5 provided semen samples. Semen analysis was performed and scored according to World Health Organization 2010 criteria (26): sperm concentration, motility, morphology, and vitality, and semen volume were assessed. Abbreviations: LRL, lower reference limit; n.d., not determined.

Testicular adrenal rest tumors

TARTs were visualized by ultrasound or MRI at cross-sectional investigation in 28/68 patients. For 1 patient, the diagnosis was based on retrospective histology data. Furthermore, retrospective imaging data were available for 11 men: TARTs were present in 10 of these individuals. In the total population screened, TARTs were present in 39/80 patients (48.8%) of which 34 were bilateral TARTs (87.2%). Documented retrospective TARTs at age 16 years were reported in 16/30 patients (53.3%), all of which were bilateral. In only 2/16 patients (12.5%) with TART reported to be present at age 16 years, TART was no longer observed during the cross-sectional investigation: 1 patient was misdiagnosed with TART as it appeared to be a varicocele, and in the other patient, TART (size 2 mm) disappeared after treatment with prednisone. This patient was still considered as a patient with TART in all analyses.

Comparison of patients with and without TART

Table 7.4 shows associations of TART with various variables in the 68 patients with gonadal imaging data (12 patients were excluded due to testosterone substitution, 11β-hydroxylase deficiency or unconfirmed 21-hydroxylase deficiency), comprising 33 patients with and 35 without TARTs. Genotype was associated with the presence of TART: The null genotype group had the highest prevalence of TART (14/18: 77.8%), while the prevalence was 10/27 (37.0%) for genotype group A and 7/21 (33.3%) for genotype group B. The odds of having TART in the null genotype group was 6.0 (1.5-23.1) and 7.0 (1.7-29.4) times higher compared to the genotype groups A and B, respectively. TARTs were also present in both men in the genotype C group and also in 1 CYP11B1-deficient patient (the other CYP11B1 patient did not undergo assessment

for TART). The OR of having TART when having a serum androstenedione concentration above the upper limit of normal at the time of the cross-sectional investigation was 3.6 (1.0-12.7). Similar associations were found for serum 17-hydroxyprogesterone at the cross- sectional investigation, with an OR of 28.0 (3.1-252.5) for having TART when 17-hydroxyprogesterone concentrations were more than twice the upper level of the reference range and an OR of 18.7 (2.2-158.1) when these concentrations were above the reference range compared to concentrations within the reference range.

Table 7.4: Comparison of genetic and hormonal characteristics between patients with (*n*=33) and without testicular adrenal rest tumors (*n*=35).

Characteristics	With TARTs	Without TARTs	OR (95% CI)
Genotype *			
Group null	14 (77.8%)	4 (22.2%)	
Group A	10 (37.0%)	17 (63.0%)	
Group B	7 (33.3%)	14 (66.6%)	
Group null - Group A			6.0 (1.5-23.1)
Group null - Group B			7.0 (1.7-29.4)
Androstenedione			3.6 (1.0-12.7)
> ref range	12 (66.7%)	6 (33.3%)	
Within ref range	9 (36.0%	16 (64.0%)	
17-hydroxyprogesterone			
>2x UL ref range	16 (66.7%)	8 (33.3%)	
> ref range	20 (57.1%)	15 (42.9%)	
Within ref range	1 (6.7%)	14 (93.3%)	
>2xUL - within			28.0 (3.1-252.5)
> ref - within			18.7 (2.2-158.1)

Odds ratios with 95% confidence intervals were calculated for many comparisons, but only odds ratios that clearly or potentially differ from unity are presented. Data are presented as number and percentage for each characteristic. *Genotype group C and *CYP11B1* category contained only 2 and 1 case(s), respectively and are not included in the analysis. Abbreviations: 95%CI, 95% confidence interval; OR, odds ratio; ref, reference; TART, testicular adrenal rest tumor; UL, upper limit.

Paternity

Data on paternity were available for 113 of the 121 patients, 26 (23.0%) of whom (age range 26-68 years) had fathered a total of 43 children. Three couples had used assisted reproductive techniques (ART) resulting in 4/43 children. One of the men who had used ART had decreased testosterone concentrations, while another had increased FSH, decreased sperm concentration and TART. No information was available about the third patient who had used ART.

Discussion

This unique and relatively large European multicenter study shows that gonadal dysfunction is a common complication in male patients with CAH. Approximately half of the patients were affected by endocrine disturbances of the HPG axis at an adult age and TARTs were present in approximately half of the patients as well.

The difficulty in diagnosing hypogonadism in men with CAH is related to the fact that testosterone measured in serum is a mixture of testosterone of gonadal and adrenal origin (25, 28). Circulating testosterone in male patients with well-controlled CAH is predominantly derived from testicular production, but when there is poor hormonal control, a relevant contribution arises from adrenal steroidogenesis. Until now, no method is able to discriminate between testosterone derived from the testes or the adrenal gland. Therefore, it has been suggested to use the serum AD/T ratio in male patients with CAH, as this precursor steroid is elevated in serum when serum androgens are predominantly of adrenal origin (25). Our data point toward an association between an AD/T ratio ≥1 (testosterone mainly of adrenal origin) and decreased LH and/or decreased FSH concentrations, suggesting that adrenal androgens in men with CAH contribute to the suppression of gonadotropins. In approximately half of the patients, either aberrant testosterone or AD/T ratios, or aberrant gonadotropin concentrations or a combination of both were found. In previous studies, the reported prevalence of endocrine HPG axis disturbances ranged from 20% to 52% (5-7, 9, 10). However, only 1 other study provided information on testosterone and gonadotropin concentrations in each patient and also indicated hypogonadism in approximately half of the patients (6). We recommend to include the evaluation of the AD/T ratio in the regular follow-up of male patients with CAH and interpret this ratio in combination with gonadotropin concentrations in order to detect a disturbance of the HPG axis. Our study does not include data on 11-oxygenated androgens, that are generated through conversion of androstenedione and are reported to be elevated in patients with CAH (29, 30). Recent studies indicate that 11-oxygenated androgens are almost entirely derived from the 11β-hydroxylation of androstenedione in the adrenal, and as they are potent androgens they can contribute to suppression of the HPG axis (31). However, their exact role in the evaluation of hormonal control and gonadal function in men with CAH has to be established in further studies. Serum AMH and inhibin B are also used as markers for male fertility (32). However, it has been demonstrated that serum AMH concentrations do not correlate with sperm concentration and other male fertility parameters (33). Serum inhibin B, a marker of Sertoli cell function, is known to correlate with spermatogenesis in healthy men (34) and was decreased in 18.6% of our cohort. Semen quality, assessed in one third of the study cohort, was reduced in 40% of the men. Except for the study of Urban *et al.* (3), all other studies on fertility in male

patients with CAH showed decreased sperm concentrations ranging from 47.8% to 66% (4-7, 9, 10). More strikingly, in all studies, only half of the participants participated in semen analysis. Taken together, these data indicate the need for increased awareness on fertility status in patients with CAH and to start performing semen analysis and gonadal function biomarkers assessment from adolescence on, in order to detect disturbances early and allow semen preservation for later fertility purposes.

Data from our cohort indicate, in agreement with previous studies (4-10, 12-22), that TART is a common complication in males with CAH (with a prevalence of 48.8%) and can have onset as early as in adolescence. In fact, 14 patients with TART at the time of the dsd-LIFE study already had TART at the age of 16 years. TARTs disappeared on treatment with prednisone in only 1 patient, thus indicating that complete regression of TART might only be achieved in a small proportion of the patients. Hence, prevention of the development of TART should be pursued, by optimizing treatment strategies already in childhood. Current standard of care does not include imaging of testes; however, we recommend incorporating testicular ultrasound in routine clinical practice.

In contrast to previous studies (4, 9, 10), we observed an association between the *CYP21A2* genotype and the presence of TARTs, with the prevalence of this complication being highest in men with the null *CYP21A2* genotype. This supports the current perception that TARTs are more frequently observed in patients with a more severe form of CAH, as these patients are exposed to higher concentrations of ACTH, already *in utero*, which is thought to be a possible causative factor for TART development (6, 7, 15, 22). However, a clinically relevant finding in this study is that TARTs occur even in less severe forms of 21OHD. In fact, in our study, 2 patients in genotype group C (both compound heterozygous for deletion and P30L mutation) had TARTs. In our current dataset, we could not find an association between genotype and semen quality or genotype and hypogonadism.

We found an association between increased 17-hydroxyprogesterone concentrations at cross-sectional data assessment and the presence of TART. Although a single 17-hydroxyprogesterone measurement may not be representative of overall metabolic control, these results could be interpreted as a possible indicator of the patient's metabolic control in the recent past. Therefore, our results seem to be in accordance with literature reporting higher TART prevalence in patients with poor hormonal control compared to patients with adequate hormonal control (5, 7, 13, 35-38). The association between increased androstenedione concentrations at cross-sectional data assessment and the presence of TARTs adds evidence to this pathophysiologic concept, even if the AD/T ratios were not clearly associated with TART within this subgroup of

patients. Primary gonadal dysfunction may be suggested by raised FSH concentrations. In our dataset, 10 patients (11.1%) had elevated FSH concentrations. Seven of these patients had data on the presence of TART and 4 had evidence of TART. King *et al.* found that testicular failure was a consequence of TART in the majority of cases (10). However, our data are limited and do not allow firm conclusions concerning this issue.

Despite this being the first international multicenter study describing gonadal function in male patients with CAH, the study also has some limitations. All centers included in this consortium are tertiary care centers; therefore, it is possible that the patient groups were selected and that the patients included were more severely affected. Furthermore, serum hormone concentrations were not measured centrally, but in various centers, with a range of different assays. Accounting for this fact, only range variables were used in the data analyses. The median BMI in our patient cohort was 25.6 kg/m^2 (range: 22.0-29.2), which is slightly overweight. It has been demonstrated that excess of total and abdominal body fat could represent one cause of fertility impairment in men with CAH (25). Serum total testosterone can be decreased in patients with obesity, as a result of the decreased serum concentration of SHBG. In case of increased serum SHBG (induced by hepatitis, hyperthyroidism or a genetic variant), total testosterone may be increased. Ideally, free testosterone should be measured in these cases, but this requires complex equilibrium dialysis (39). Free testosterone can also be calculated from total testosterone, SHBG, and albumin concentrations, but it is crucial that the results of such calculations are compared with the normal range of each separate laboratory. Such data were not available. We are aware that assessment of fertility by paternity numbers in our study was incomplete, as many other factors, including female fertility, were not available. Furthermore, participation in the medical examination was not compulsory for study inclusion. This may have led to even more selection, especially concerning the ultrasound examination and semen analysis. It is likely that only the very motivated patients and the more severely affected patients consented to these additional examinations. Due to the resulting low numbers of available data, multivariable logistic regression analyses were not possible.

In summary, impaired gonadal function is common in adult men with CAH. This is indicated by the presence of TART and/or hypogonadotropic or hypergonadotropic hypogonadism. The risk of TART is highest in men with the most severe enzyme deficiencies underlying CAH. Our data suggest that an association with poor previous hormonal control is likely but requires confirmation by prospective studies. Determination of the serum AD/T ratio, in addition to serum concentrations of testosterone, androstenedione, LH, and FSH may help to differentiate between testicular and adrenal androgens in male patients with CAH and to diagnose gonadal

dysfunction. Routinely performed semen analysis, measurement of serum inhibin B and testicular ultrasound investigation already in adolescence are recommended to detect upcoming reproductive problems and to allow for fertility preserving measures, such as sperm banking.

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Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this clinical study.

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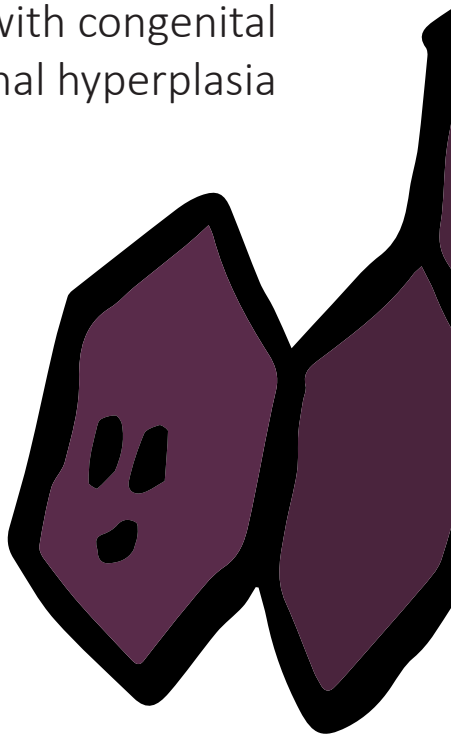
Chapter 8

Quality of life in adult males with congenital adrenal hyperplasia



Engels M[1,2,], Verhees MJM[1,*], Span PN[3], Sweep FCGJ[2], van Herwaarden AE[2], Falhammar H[4,5], Nordenström A[6], Webb EA[7,8], Richter-Unruh A[9], Roeleveld N[10], Bouvattier C[11], Brac de la Perrière A[12], Arlt W[7,8], Reisch N[13], Köhler B[14], Rapp M[15], Stikkelbroeck NMML[16] and Claahsen-van der Grinten HL[1] on behalf of the dsd-LIFE group*

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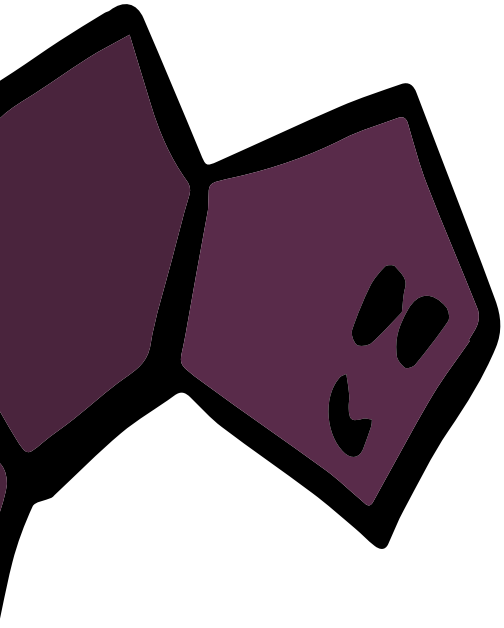

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Submitted

Abstract

Objective: Congenital Adrenal Hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) is a disorder of adrenal steroid biosynthesis, leading to hypocortisolism, hypoaldosteronism, and hyperandrogenism. Impaired quality of life (QoL) has been demonstrated in females with CAH, but data in males with CAH is scarce. We hypothesize that disease severity and poor treatment control are inversely associated with QoL.

Design: European multicenter, cross-sectional study: 'dsd-LIFE'.

Methods: Adult (16-68 years) males with 21OHD were included (n=109). The WHOQOL-BREF questionnaire was used to measure self-reported QoL domain scores on a 0-100 scale, where higher scores reflect better QoL. QoL domain scores were compared to published data on healthy and chronically ill reference populations from Germany, France, the Netherlands, and the United Kingdom. Associations between QoL and disease severity and poor treatment control were tested (Mann-Whitney U test).

Results: Median domain scores were 78.6 (IQR: 67.9-85.7 physical health), 79.2 (IQR: 66.7-87.5 psychological health), 75.0 (IQR: 58.3-83.3 social relationships) and 81.3 (IQR: 71.9-90.6 environment). Overall, these scores were similar to WHOQOL-BREF domain scores in healthy references and higher compared to chronically ill reference populations. Domain scores did not differ between genotype groups, while patients with undertreatment or increased 17-hydroxyprogestrone concentrations scored significantly higher on QoL ($p<0.05$). Significantly higher QoL domain scores were observed in patients on prednisone and dexamethasone ($p<0.05$).

Conclusions: Males with CAH appear to rate their QoL as good as healthy, and better than chronically ill, reference populations. The use of prednisone or dexamethasone and undertreatment were associated with higher QoL domain scores.

Introduction

Congenital Adrenal Hyperplasia (CAH) is an inherited disorder of adrenal steroid biosynthesis. The most common cause is a mutation in the *CYP21A2* gene leading to 21-hydroxylase deficiency (21OHD), which results in impaired production of cortisol and increased production of adrenal androgens leading to virilization of the female external genitalia. Aldosterone production is also impaired to a variable degree, depending on the severity of the enzyme deficiency (1, 2). Treatment consists of glucocorticoid substitution and, if necessary, also substitution of mineralocorticoids. Common long-term complications are disturbed pubertal development, reduced final height, decreased bone mineral density, decreased reproductive function including testicular adrenal rest tumor development, and an increased risk on obesity, cardiovascular morbidity and adrenal crises (3-5).

In patients with CAH, several factors may affect quality of life (QoL), such as the development of long-term complications. The use of medication and poor treatment control may also contribute to impaired QoL. Impaired QoL has been reported in CAH, but mostly in women (reviewed by Reisch *et al.* (3)). Data on QoL in men with CAH are scarce, although separate analysis of QoL in male and female patients is important since clinical presentation and complications vary greatly between the 2 sexes. Results are contradictory with some papers describing impaired QoL (6-8), and others equal (9) or better (10) QoL in males with CAH compared to a control population.

The dsd-LIFE study provides a unique opportunity to study a large European multicenter cohort of males with CAH. We aimed to analyze QoL in males with CAH using the WHOQOL-BREF questionnaire and to assess factors that may contribute to QoL.

Subjects and methods

Subjects

Adult males with CAH were included from the dsd-LIFE study, a cross-sectional clinical outcome study of individuals with disorders/differences of sex development (DSD). Fourteen study centers in 6 European countries (France (n=4), Germany (n=4), United Kingdom (UK) (n=1), Poland (n=2), Sweden (n=1), and the Netherlands (n=2)) included participants with DSD, from February 2014 until September 2015. In addition, 121 males with CAH (46XY karyotype) aged 16-68 years, not classified as DSD patients, were included as an add-on study, as these patients might face similar challenges as patients

with DSD. Written informed consent was obtained from all participants. The study was approved by the medical ethics committee at the Charité Universitätsmedizin Berlin (reference number EA2/069/13) and the local ethics committees of the other study centers (Ethik-Kommission der Universität zu Lübeck (13-144), Ethik-Kommission der Medizinischen Fakultät der Westfälischen Wilhelms-Universität Münster (2013-500-b-S), Ethik-Kommission bei der LMU München (450-13), Comite de Protection des Personnes-Ile de France 1 (13352), Komisja Bioetyki Uniwersytetu Medycznego w Lodzi (RNN/242/13/KE), Komisja Bioethycnej przy intytucie 'Pomnik-centrum Zdrowia Dziecka (103/KBE/2013), Regionala etikprövningsnämnden I Stockholm (2013/1163-31/1), Medisch Ethische Toetsingscommissie VU medisch centrum (2013.336), Radboud universitair medisch centrum (NL46220.029.13), NHS National Health Research Ethics Service, NRES Committee North West-Greater Manchester East (14/NW/1123)). The methodological background of the dsd-LIFE study was described previously (11). The same cohort of males with CAH was included previously in a study reporting on gonadal function (12).

Patients were investigated in their local medical center. All patients underwent medical examination and filled out several questionnaires, including the WHOQOL-BREF (13). Additional data were retrieved from medical records. General patient characteristics and clinical parameters included: country of inclusion, height, weight, BMI, age, age at diagnosis, CYP21A2 genotype, medication use, subjective treatment control, satisfaction with care in childhood, smoking behavior, work, life activity, sports, and educational level. The variable age was dichotomized into <30 years and ≥30. The patients were classified into genotype groups null (0), A, B, and C (14), as described earlier (12). The patients with genotype group 0 are most severely affected, while genotypes A, B, and C have decreasing severity. The patients' educational levels were established according to the EU classification as low, medium, and high as described earlier (12).

WHOQOL-BREF

To assess QoL in this study, the WHOQOL-BREF questionnaire was used according to guidelines provided by the World Health Organization (13). The WHOQOL-BREF is a shortened version of the WHOQOL-100, consisting of 24 questions concerning QoL on 4 different domains: physical health, psychological health, social relationships, and environment. Questions are rated on a 5-point Likert scale and domain scores represent the mean score of items within each domain. Scores are multiplied by 4, resulting in a score ranging from 4-20, to be directly comparable with scores derived from the WHOQOL-100. These scores can then be converted to a 0-100 scale. High scores reflect good QoL. Healthy as well as chronically ill reference populations available from Germany(15), France (16), the Netherlands (17), and the UK (18) were

used for comparison. Reference populations form the Netherlands and the UK contained both men and women, as Germany and France reported gender-specific QoL scores.

Medication

All glucocorticoid preparations were converted to hydrocortisone equivalents per day, as described earlier (12) using the following factors for the glucocorticoid equivalent dose: 1 (hydrocortisone), 4 (prednisone or prednisolone), 30 (dexamethasone), and 15 (fludrocortisone) (19).

Treatment control

Treatment accuracy was estimated by the treating physicians in subjective scores: undertreatment, accurate treatment, or overtreatment. Blood hormone concentrations can also indicate treatment accuracy as high concentrations of androstenedione and 17-hydroxyprogesterone indicate inadequate adrenal androgen suppression. Therefore, blood samples were obtained at study inclusion. Samples were mostly taken in the morning, before intake of the glucocorticoid medication (11). Androstenedione and 17-hydroxyprogesterone concentrations were measured in the local hospital laboratory and compared to local reference values. Results were reported as “below reference range”, “within reference range”, “above reference range up to twice the upper limit", and "more than twice the upper limit of the reference range”. To increase the number of patients per category, we combined the latter 2 categories into the category ‘above reference range’.

Statistical Analysis

SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA) was used for all analyses. First, missing data were evaluated for each variable. Following, descriptive analyses were performed for all variables. After checking the distributions of the QoL scores for normality, median and interquartile ranges (IQR=Q1-Q3) were calculated. Comparative analyses were performed when subgroups contained at least 5 cases for QoL domain scores and age, age at diagnosis, CYP21A2 genotype, medication use, treatment control, smoking behavior, work, life activity, sports, and educational level. Differences were assessed using the Mann-Whitney U (MWU) test or Spearman’s correlation for categorical and continuous variables, respectively. P-values less than 0.05 were considered as statistically significant.

Results

Subjects

After exclusion of 10 males with CAH who did not complete the WHOQOL-BREF and 2 males with 11β-hydroxylase deficiency, 109 males with CAH due to 21-hydroxylase deficiency were included in this study on QoL. General characteristics are shown in Table 8.1. The median age of the study population was 29 years (range 16-68, IQR 21-41). Educational level was low in 14 (14.3%), medium in 59 (60.2%), and high in 25 (25.5%) patients. Genotypes 0, A, B, and C comprised 20.2%, 30.3%, 27.5%, and 2.8% of the participants, respectively. In 21 males with clinical and biochemically proven CAH

Table 8.1: General characteristics of 109 adult males with congenital adrenal hyperplasia due to 21-hydroxylase deficiency.

Variable	N		Median (IQR) or number (%)
Age	109		
Country of inclusion	109	Germany	46 (42.2%)
		France	30 (27.5%)
		The Netherlands	12 (11.0%)
		United Kingdom	12 (11.0%)
		Sweden	9 (8.3%)
Height	108		170.0 (116.3-175.0) cm
BMI	108		25.4 (22.6-29.8) kg/m^2
Education level	98	Low	14 (14.3%)
		Medium	59 (60.2%)
		High	25 (25.5%)
Genotype	109	Group 0	22 (20.2%)
		Group A	33 (30.3%)
		Group B	30 (27.5%)
		Group C	3 (2.8%)
		No mutation reported	21 (19.2%)
Glucocorticoids	109	No glucocorticoids	5 (4.6%)
		Hydrocortisone	63 (57.8%)
		Prednisolone	15 (13.8%)
		Prednisone	12 (11.0%)
		Dexamethasone	7 (6.4%)
		More than one glucocorticoid*	7 (6.4%)
Glucocorticoid dose**	108		27.9 (22.3-31.5) mg / day
Mineralocorticoids	109	Fludrocortisone	79 (72.5%)
		No fludrocortisone	30 (27.5%)
Fludrocortisone dose	79		100.0 (75.0-150.0) mcg/day

Continuous variables are displayed as median (IQR: Q1-Q3). Categorical variables are displayed as number of participants with percentage. Patients were classified according to severity of the disease into genotype group null (0) to group C (14). Abbreviations: 21OHD, 21-hydroxylase deficiency; BMI, body mass index; IQR, interquartile range. *6 patients used hydrocortisone and dexamethasone, and 1 patient hydrocortisone and prednisolone. **Hydrocortisone equivalent scores were calculated for the total dose of glucocorticoids used per day (19).

(19.2%), no mutation was reported. Glucocorticoids were used by 104 (95.4%) of the participants: most commonly hydrocortisone (57.8%), followed by prednisolone (13.8%), prednisone (11.0%), and dexamethasone (6.4%). In this cohort, 7 males used 2 different glucocorticoids (hydrocortisone and dexamethasone (n=6) or hydrocortisone and prednisolone (n=1)). Overall, median glucocorticoid dose in hydrocortisone equivalents was 27.9 (IQR: 22.3-31.5) mg/day (n=108). Fludrocortisone was used by 79 participants (72.5%) with a median dosage of 100.0 (IQR: 75.0-150.0) mcg/day.

QoL domain scores

Overall, males with CAH rated their QoL with median domain scores of 78.6 (IQR: 67.9-85.7), 79.2 (IQR: 66.7-87.5), 75.0 (IQR: 58.3-83.3), and 81.3 (IQR: 71.9-90.6) for physical health, psychological health, social relationships, and environment, respectively (Table 8.2). Median scores appeared to be lowest on all domains in UK males, and highest in Dutch males (Table 8.2). Males <30 years (n=56) gave significantly higher scores (median: 75.0, IQR: 60.4-91.7) compared to males ≥30 years (n=53, median: 66.7, IQR: 50.0-83.3) on the social relationships domain (MWU: p=0.028). Highly educated males gave significant lower scores (median: 70.8, IQR: 60.4-83.3) on the psychological domain compared to medium educated males (median: 79.2, IQR: 70.8-87.5, p=0.049). No significant differences were observed on the other QoL domains for age or education level.

Physical health and social relationships domain scores appeared to be similar in the total cohort of males with CAH compared to healthy subjects from Germany, France, and the UK (Table 8.2). On the psychological health domain, the total group of males with CAH appeared to have a similar score compared to the healthy reference population from Germany, although seemingly higher QoL scores were observed compared to healthy references from France, the Netherlands, and the UK (Table 8.2). Country-specific analyses indicated that males with CAH from France and the Netherlands rated their psychological health higher compared to the corresponding healthy reference populations, while males with CAH from the UK had lower scores compared to the corresponding healthy references. On the environmental domain, the total cohort of males with CAH appeared to report higher scores compared to healthy reference populations from Germany, the Netherlands, and the UK. Regarding the country-specific domain scores, males with CAH from Germany and the Netherlands rated their QoL on the environmental domain seemingly higher compared to reference populations from the corresponding countries, whereas males with CAH from the UK had similar scores as the healthy UK reference population.

In comparison to chronically ill reference populations from Germany, France, and the UK (chronic endocrine disorders), higher scores were reported in the total cohort of

Table 8.2: Quality of Life domain scores in adult males with CAH due to 21-hydroxylase deficiency and country specific reference populations.

	Total cohort	Germany*			France			The Netherlands		United Kingdom			Sweden
Domain	CAH n=109	CAH n=46	healthy n=925	ill^ n=261	CAH n=30	healthy n=5157	ill n=1638	CAH n=12	healthy^ n=218	CAH n=12	healthy^ n=1328	ill^ n=524	CAH n=9
Physical health	78.6 (67.9 - 85.7)	83.9 (75.0-92.9)	78.8	53.4	71.4 (63.4-78.6)	81.6	68.4	89.3 (66.1-96.4)	70.1	58.9 (45.5-85.7)	76.5	67.8	82.1 (67.9-85.7)
Psycho-logical health	79.2 (66.7 - 87.5)	79.2 (70.8-87.5)	75.9	62.7	77.1 (66.7-87.5)	69.5	67.5	81.3 (59.4-91.7)	64.8	60.4 (32.3-72.9)	67.8	67.7	79.2 (66.7-81.3)
Social relation-ships	75.0 (58.3 - 83.3)	75.0 (58.3-83.3)	72.3	68.0	66.7 (50.0-75.0)	75.6	71.8	87.5 (68.8-100.0)	71.3	41.7 (10.4-75.0)	70.5	70.1	66.7 (50.0-79.2)
Environ-ment	81.3 (71.9 - 90.6)	84.4 (75.0-90.6)	71.2	67.2	76.6 (71.1-82.0)	-	-	89.1 (67.2-96.9)	74.0	65.6 (52.3-78.1)	68.2	71.1	81.3 (68.8-93.8)

Median WHOQOL-BREF domain scores and interquartile range (Q1-Q3) from the overall cohort and from country-specific analysis were calculated. Median domain scores were compared to mean domain scores of country-specific reference populations from the literature (15-18). If available, both healthy and chronically ill reference populations were used. Chronically ill reference populations comprised patients with a chronic physical illness: in the United Kingdom only patients with a chronic endocrine disorder were included. *The German healthy reference populations contains all male patients from the cohort including chronically ill patients. ^Quality of life scores were obtained from a reference population containing both men and women.

males with CAH compared to patients with other physical chronic diseases in these reference studies. An exception was the social relationships domain where reference patients from France and the UK had comparable scores. Country-specific analyses also indicated lower median scores in males with CAH from the UK (physical health and social relationships) compared to corresponding chronically ill reference population.

QoL domain scores in different genotypes

QoL domain scores were not significantly different between the different genotype groups (Fig. 8.1A-D, MWU: $p>0.05$). Interestingly, median QoL domain scores seemed among the highest on all 4 domains in males with genotype 0, who are most severely affected.

Associations of medication use and treatment control with QoL domain scores

No clinically relevant correlations were observed between glucocorticoid dose in hydrocortisone equivalents and median QoL domain scores. Significantly higher domain scores were observed in patients on prednisone and dexamethasone on the physical health, psychological health and social relationships domain (see Fig. 8.2A-D). No significant differences between the glucocorticoids types were observed in the

environmental domain. Patients who were treated with fludrocortisone had significantly lower QoL scores on the physical health domain (n=79, median: 78.6, IQR: 64.3-85.7) compared to patients that did not receive fludrocortisone (n=30, median: 82.1, IQR: 74.1-92.9, MWU: p=0.033), although fludrocortisone dose was not correlated.

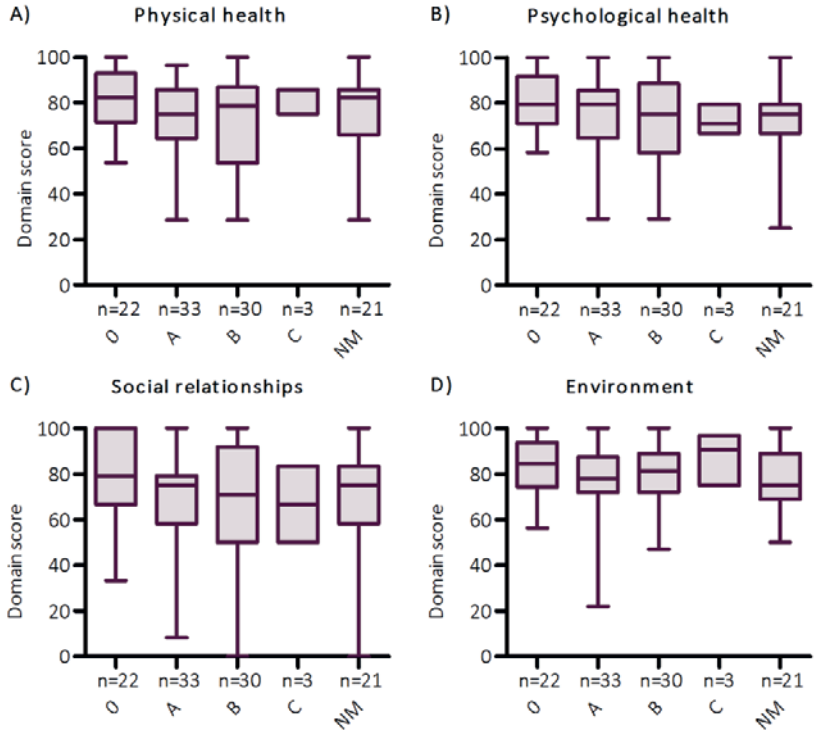

Fig 8.1: Quality of Life domain scores in genotypes. Patients with 21-hydroxylase deficiency were classified into genotype group 0, A, B, or C (14). Genotype C was excluded from comparative analysis due to low patient numbers (n=3). WHOQOL-BREF scores in different CAH genotype groups were calculated for 4 different domains: **A)** physical health, **B)** psychological health, **C)** social relationships, and **D)** environment. Boxes represent median and 25th-75th percentile, while whiskers show minimum-maximum domain scores. Differences between groups were assessed using Mann-Whitney U test, where $p<0.05$ was considered significant. Abbreviations: NM, no mutation reported.

Subjective treatment accuracy differed significantly as patients with undertreatment (n=13) had significantly higher domain scores on the social relationships (median: 83.3, IQR:70.8-100.0, MWU: p=0.037) and environmental domain (median: 87.5, IQR: 79.7-95.3, MWU: p=0.010) compared to patients on accurate treatment (n=71, median:

75.0, IQR: 58.3-83.3, and median: 81.3, IQR: 71.9-87.5, respectively). On the other 2 domains, no differences were observed. Furthermore, patients with 17-hydroxyprogesterone concentrations above reference range (n=57), indicating inadequate adrenal suppression, reported significantly higher domain scores on the psychological health (median: 79.2, IQR: 70.8-87.5, MWU: p=0.011) and environmental

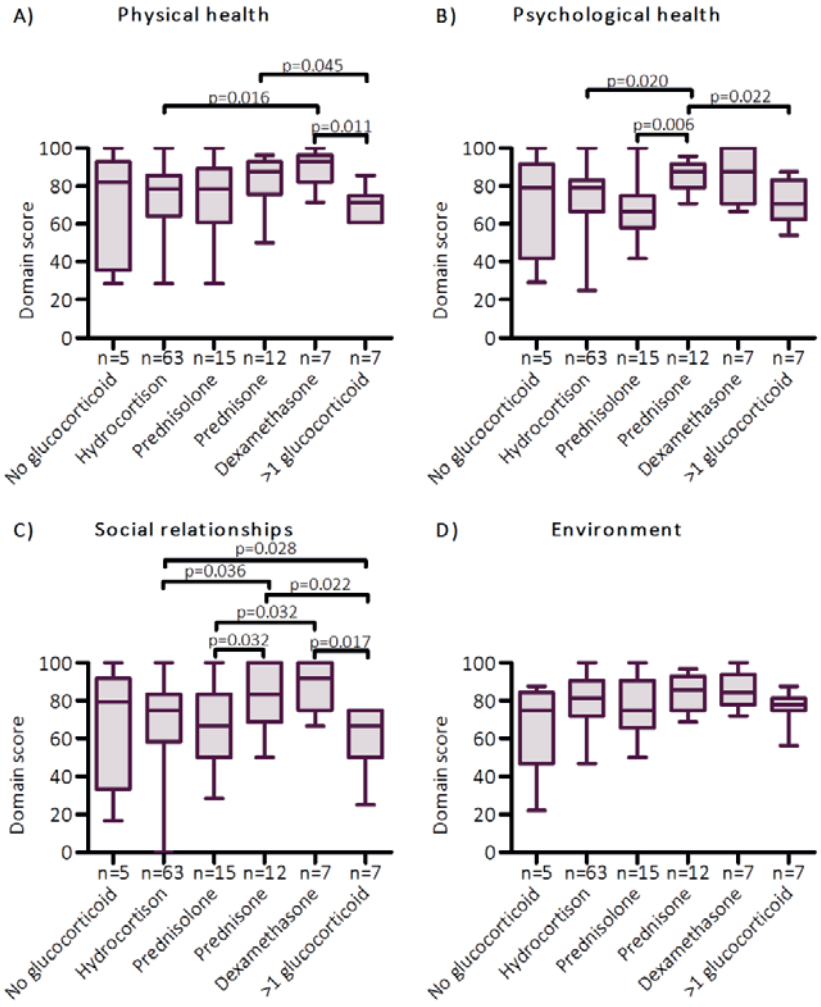

Fig. 8.2: Quality of Life domain scores in adult males with CAH due to 21-hydroxylase deficiency divided by glucocorticoid type. WHOQOL-BREF scores in different glucocorticoid type groups were calculated for 4 different domains: **A)** physical health, **B)** psychological health, **C)** social relationships, and **D)** environment. Boxes represent median and 25th-75th percentile, while whiskers show minimum-maximum domain scores. Differences between groups were assessed using Mann-Whitney U test, where $p<0.05$ was considered significant.

domain (median: 81.3, IQR: 75.0-90.6, MWU: p=0.049) compared to males with 17-hydroxyprogesterone concentrations within reference range (n=23, median: 70.8, IQR: 58.3-79.2, and median: 78.1, IQR: 68.8-81.3, respectively). No significant differences in QoL domain scores were found for androstenedione concentrations.

Discussion

This is the first international multicenter study examining QoL using the WHOQOL-BREF questionnaire in adult males with CAH. This study shows that males with CAH rate their QoL as good, with median domain scores between 75.0 and 81.3. Overall, these scores appeared to be similar to scores obtained with the WHOQOL-BREF questionnaire in healthy reference populations from Germany (15), France (16), and the UK (18), and higher compared to a healthy reference population from the Netherlands (17). We also compared our results to chronically ill reference populations, as having a chronic disease may affect expectations of life, leading to higher QoL scores due to overrating (20). These data show that males with CAH appeared to rate their QoL higher compared to patients with other chronic diseases.

Although adult males with CAH may face different complications of their chronic disease and often require lifelong therapy, they do not report a worse QoL on the physical health domain compared to healthy or chronically ill references. On the psychological health domain, males with CAH also report similar or higher QoL scores compared to reference populations. Although lower QoL scores were found in highly educated males, these males still reported good psychological health with a median QoL score of 71. These findings suggest that psychological wellbeing does not seem to be largely affected in males with CAH, which is in contrast to previously reported higher prevalence of psychiatric morbidity in males with CAH (21). However, patients with mental health issues might be less likely to participate in studies or fill out questionnaires on QoL, and might be underrepresented in the dsd-LIFE database. Social relationships as well as environmental QoL domain scores appeared to be higher in males with CAH compared to reference scores. Higher QoL domain scores were observed in patients with CAH < 30 years old compared to patients ≥ 30 years on the social relationships domain. This is supported by Skevington *et al.*, who report decreasing WHOQOL-BREF domain scores with increasing age in a large healthy international cohort (22).

In country-specific analyses, we observed high scores on all 4 domains in the Dutch males with CAH compared to males with CAH from other countries. This result is not reflected in the reference literature, as the Dutch healthy reference population had the

lowest scores on 3 out of 4 domains compared to reference populations from the other countries (15-18). Possibly, the Dutch reference study does not accurately reflect the current Dutch general population as the data were collected 15 years earlier than the dsd-LIFE study from a matched control group. Another notable finding in the country-specific analyses was that males with CAH from the UK reported low scores on all 4 domains. Compared to healthy references from the UK (18), males with CAH rated QoL with rather comparable scores on the environmental domain, while scores seemed lower on the physical health, psychological health, and social relationships domains. However, the healthy reference population from the UK consisted of both male and female university students and student nurses, resulting in a most likely young and highly educated cohort. This may have led to overestimation of the QoL scores of the general population.

Few other studies have reported QoL in males with CAH, and these studies used different questionnaires than the WHOQOL-BREF questionnaire to assess QoL. Our results indicate a good QoL in males with CAH, which corresponds to the results found by Falhammar *et al.* (9), although impaired QoL has also been reported (6-8). Strikingly, Reisch *et al.* showed impaired QoL in males with CAH on the GBB-24, while QoL measured with the HADS and SF-36 did not differ from a healthy reference population (8). This stresses the importance of using similar questionnaires to assess QoL in patients with CAH to make comparison between different study populations possible. As no disease-specific questionnaires are available, we propose the use of the WHOQOL-BREF questionnaire as it provides a broad aspect of self-perceived QoL on different QoL domains.

QoL in males with CAH in our study was also higher compared to patients with primary adrenal insufficiency, although QoL in this study was not measured by WHOQOL-BREF (8). Furthermore, QoL measured by WHOQOL-BREF in males with CAH was higher compared to patients with DSD, including Turner Syndrome, Klinefelter syndrome, XY female DSD, and XY male DSD (23). One of the differences between these diseases is the presence of increased adrenal steroid precursors in CAH, in contrast to patients with primary adrenal insufficiency or DSD. Previously, we showed that several adrenal steroid precursors that are elevated in CAH patients are able to activate the glucocorticoid receptor, which might explain why patients with CAH experience fewer complications of their cortisol deficiency than expected (24), possibly leading to better QoL. The QoL observed in males with CAH is also higher compared to QoL observed in females with CAH (23). This may reflect the difference in clinical presentation and complications, as female patients have more problems due to increased adrenal androgens, such as virilization, masculinization and consequently corrective surgery, which may affect QoL negatively.

QoL domain scores did not differ between the different genotypes, confirming the findings in a previous study (9). However, we did find that fludrocortisone therapy, given to the most severely affected CAH patients, was associated with lower QoL scores on the physical domain. Remarkably, males with genotype 0, who are most severely affected, had high median QoL scores. Possibly, altered expectations of life are more pronounced in this group of patients, who received their diagnosis directly postnatal. It can be speculated that an early start of follow-up postnatal may have led to improved QoL.

In accordance to previous studies (8, 9), we did not find an association between glucocorticoid equivalent dose and median QoL domain scores. However, patients on prednisone or dexamethasone rated QoL significantly higher compared to patients that used other types of glucocorticoids on the physical health, psychological health and social relationships domain. In contrast, Han *et al.* reported worse QoL in patients using dexamethasone (25). However, patients included in the dexamethasone group could have been on any regimen including dexamethasone mono-therapy, as well as multiple glucocorticoids, which may have influenced the QoL scores negatively. In addition, our dexamethasone group only contained 7 males, which might have skewed the results positively. Males with CAH using multiple glucocorticoids scored among the lowest on physical health and social relationships domain. Possibly, patients with poor hormonal control are more likely to be eventually treated with more than 1 glucocorticoid. We observed higher scores in patients who were undertreated according to the subjective rating of the treating physician, as well as patients with increased 17-hydroxyprogesterone concentrations compared to patients on accurate treatment. In addition, Falhammar *et al.* reported significantly better QoL on PGWB questionnaire in males with CAH in undertreated compared to overtreated patients (9). It can be hypothesized that male patients with CAH suffer less from androgen excess due to undertreatment than from glucocorticoid excess in overtreatment.

Although we were able to include a large cohort of adult males with CAH, our study design did not include a reference population. Therefore, we compared our domain scores with scores reported in literature, although only mean domain scores were available and only the German and France cohort reported gender-specific QoL scores. This complicated the comparison to our median domain scores. Until now, no cut-off values for 'good QoL' have been described in literature for the WHOQOL-BREF. Although we set a minimum of 5 cases per group, subgroups were still relatively small. Furthermore, it should be taken into account that all centers involved in the dsd-LIFE study are tertiary care centers. This may have led to selection of the patients groups, including more severely affected patients. However, this substantiates our finding of

good QoL in patients with CAH even more. In contrast, selection of highly motivated patients may have occurred, leading to an overestimation of QoL.

In conclusion, adult males with CAH in this study rated their QoL as good. Their QoL domain scores appeared to be comparable to healthy reference populations and higher compared to patients with a chronic illness. QoL was not influenced by genotype, while undertreatment and use of prednisone or dexamethasone led to higher QoL.

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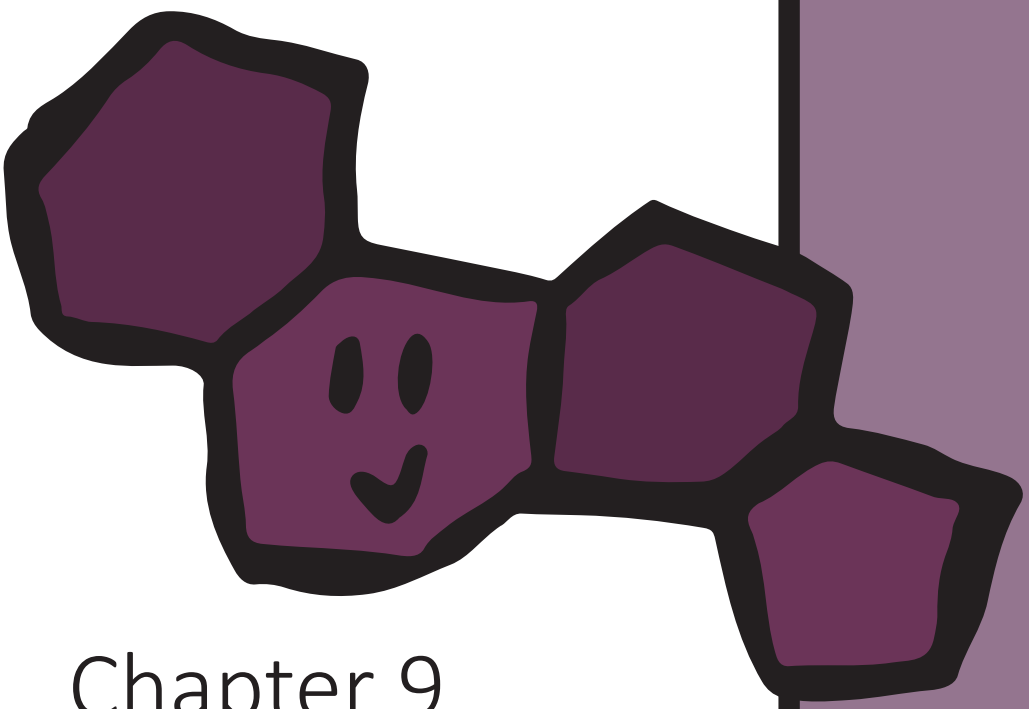

Chapter 9

General discussion and summary

The adrenal glands have important endocrine functions in our body. They are responsible for the production of steroid hormones and catecholamines, thereby regulating important functions, such as the stress response. The adrenal cortex produces three types of steroid hormones: mineralocorticoids, glucocorticoids, and adrenal androgens. Mineralocorticoids regulate our blood pressure and electrolyte balance, glucocorticoids regulate our glucose metabolism and immune response, and adrenal androgens, leading to virilization of the female external genitalia. The production of these hormones is tightly regulated by the renin-angiotensin-aldosterone system and the hypothalamus-pituitary-adrenal (HPA) axis.

Congenital adrenal hyperplasia (CAH) is a group of rare inherited disorders of the adrenal cortex, that have decreased cortisol production and consequently increased adrenocorticotropic hormone (ACTH) concentrations leading to hyperplasia of the adrenal cortex in common. CAH is predominantly caused by an enzymatic defect of 21-hydroxylase (*CYP21A2* mutations), but other enzymatic defects, such as 11-hydroxylase, 17-hydroxylase, 3β-hydroxysteroid dehydrogenase type 2, steroidogenic acute regulatory protein, P450 cholesterol side-chain cleavage enzyme, and P450 oxidoreductase, are also known to result in CAH. In addition to cortisol deficiency and increased ACTH concentrations, 21-hydroxylase and 11-hydroxylase deficiency lead to accumulation of the precursor steroids before the enzymatic defect, and increased production of adrenal androgens (1, 2).

Patients with CAH due to 21-hydroxylase deficiency suffer from the hormonal imbalance caused by increased ACTH and androgens, and decreased cortisol and aldosterone concentrations, and are at risk to develop adrenal crises and long-term complications, such as impaired gonadal function. This thesis aimed to gain further insights into the disturbed adrenal steroidogenesis and its long-term consequences in patients with CAH.

The role of adrenal steroid precursors in congenital adrenal hyperplasia

Cortisol deficiency can lead to severe complaints, such as weakness, fatigue, and weight loss, and also life-threatening adrenal crises especially in situations of increased demands of cortisol (3, 4). To prevent adrenal crises in CAH patients, lifelong treatment with glucocorticoids is indicated. Patients receive instructions to increase glucocorticoid dosages in moments of severe stress, such as surgery or severe illness, as this increases the demand for cortisol and the risk of adrenal crises (1). However, CAH patients appear to have less clinical signs of cortisol deficiency than expected

based on their enzyme deficiency (5-9). Donaldson *et al.* reported that only a minority of the CAH patients with the salt-wasting type (the most severely affected patients without residual cortisol production) develops hypoglycemia or jaundice, two typical signs of cortisol deficiency (5). Furthermore, our research group reported a salt-wasting CAH patient that was only treated with salt during the first two years of his life. During this time, he did not develop an adrenal crisis, not even during severe stress-situations, such as surgery (6). Without neonatal screening, which is nowadays implemented in many countries, patients with the simple virilizing type of CAH mostly present to the clinic with complaints related to androgen excess although their cortisol production is severely impaired. However, no history of adrenal crises has been reported in these patients (7). Some reports have also shown that the reported non-classic untreated CAH patients (less severely affected) generally do not develop adrenal crises, not even in severe stress-situations despite suboptimal cortisol response after ACTH stimulation (8, 9). In **chapter 3** we have described a cohort of long-time untreated CAH patients with severe CAH and biochemically confirmed cortisol deficiency. Although about half of these patients had experienced episodes of severe stress, none of them reported any signs of adrenal crisis. These studies indicate that in CAH patients the expected biochemical diagnosis of cortisol deficiency is not always accompanied by the expected clinical complaints.

In **chapter 2 and 3** of this thesis we proposed that the glucocorticoid activity of the increased concentrations of steroid precursors of cortisol and aldosterone in CAH patients might explain the lack of clinical signs of cortisol deficiency. Mineralocorticoids and glucocorticoids exert their function by binding to their receptor, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), respectively. After binding of the steroid in the cytoplasm, the receptor-steroid complex translocates to the nucleus of the cell. The receptor-complex can then activate gene transcription by binding to a hormone response element in the promotor region of the specific gene, initiating transactivation (10). Steroid cross-reactivity is a well-known phenomenon and can be explained by the high degree of homology of the DNA binding domain of the GR and MR (11). In **chapter 2** we have described that 21-deoxycortisol is able to bind to the GR, causing nuclear translocation and transactivation of the GR with similar potency to cortisol. 17-hydroxyprogesterone and progesterone also caused transactivation of the GR but with lower potency compared to cortisol, whereas androstenedione was not able to bind, translocate or transactivate the GR. In **chapter 3** we confirmed this potency of 21-deoxycortisol, 17-hydroxyprogesterone, and progesterone to activate the GR and studied the potency of other mineralocorticoid and glucocorticoid steroid precursors. These results are in line with few other studies that determined the potency of some steroids to activate the GR. Four studies report that the potency of aldosterone is lower than that of cortisol (12-15) and two of these

studies report that 11-deoxycorticosterone has similar potency to aldosterone (13, 14). Rupprecht *et al.* furthermore found that progesterone had similar potency to aldosterone and 11-deoxycorticosterone (13). One study only compared progesterone and 17-hydroxyprogesterone and found similar potency to activate the GR (16). Corticosterone and 11-deoxycortisol had somewhat lower potency to active the GR compared to cortisol but higher potency compared to aldosterone and 11-deoxycorticosterone (14). The potency of pregnenolone, 17-hydroxypregnenolone, 21-deoxycortisol, 11-hydroxyprogesterone, and 16-hydroxyprogesterone to activate the GR has never been studied before. In chapter 3 we compared the structure-activity relationship of the steroid precursors of cortisol and aldosterone (Fig. 9.1). This revealed that 3β-hydroxysteroid dehydrogenation is a prerequisite for a steroid to activate the GR. We also found that the most potent steroids are 11-hydroxylated, which was suggested earlier (14, 17).

CAH patients are known to have a hormonal imbalance with low cortisol and aldosterone concentrations, high androgen concentrations and accumulation of precursor steroids before the enzymatic defect. 17-hydroxyprogesterone concentrations in a 21-hydroxylase deficient (21OHD) patient can be increased over 100x the normal reference value and are therefore used in the diagnosis of CAH. In chapter 3 we indeed found that median concentrations, as measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), of 21-deoxycortisol (only measurable in 21OHD patients), 17-hydroxyprogesterone (127x higher), and progesterone (29x higher) were higher in 21OHD patients compared to controls.

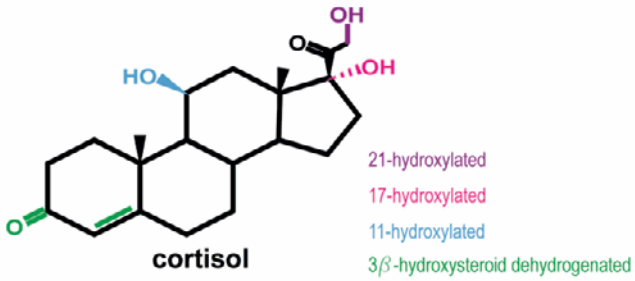

Fig. 9.1: Glucocorticoid activity is determined by the presence and absence of several molecular bonds. The molecular structure of cortisol, the main glucocorticoid, is presented. 3β-hydroxysteroid dehydrogenation (in green) is a prerequisite to enable a steroid to activate the glucocorticoid receptor (GR). Dehydrogenation alone, or in combination with 21-hydroxylation or 17-hydroxylation results in relatively low potency to activate the GR. Combination of dehydrogenation and hydroxylation of position 17&21, 11&21, or 11&17 increased the potency to activate the GR, whereas the latter two combinations had similar potency as cortisol to activate the GR. This indicates the importance of 11-hydroxylation (in blue).

In 11-hydroxylase deficient patients higher concentrations of 11-deoxycortisol (457x higher) and 11-deoxycorticosterone (55x higher) were found. These results show that steroid precursors before the enzymatic defect accumulate. All these steroids were able to transactivate the GR with similar or somewhat lower potency compared to cortisol. Therefore these steroid precursors might compensate for the cortisol deficiency.

Currently, CAH patients are treated with glucocorticoids to substitute cortisol and consequently lowering ACTH, thereby lowering adrenal steroid precursor concentrations. Turcu *et al.* showed that concentrations of 17-hydroxyprogesterone, progesterone and 21-deoxycortisol in treated 21OHD patients were still higher compared to healthy controls (18). Comparison of these concentrations in treated 21OHD patients with the concentrations in our cohort of untreated 21OHD patients (described in chapter 3), showed lower concentrations of 17-hydroxyprogesterone, progesterone, and 21-deoxycortisol in treated 21OHD patients. This indicates that therapy indeed lowers steroid precursor concentrations and suggests that the compensatory mechanism of adrenal steroid precursors will decrease due to glucocorticoid treatment, possibly making CAH patients more vulnerable to adrenal crisis as they become highly dependent on their medication. This is especially the case in severe stress-situations were normal glucocorticoid dosages are not sufficient and stress dosages are needed to prevent an adrenal crisis. For this reason it is very important that patients are extensively informed on the risk of adrenal crises and the importance of therapy compliance and stress dosages. However, in developing countries, medication is not always available and sudden discontinuation of treatment will increase the risk on adrenal crises, as the patient has no cortisol substitution anymore and cannot compensate for this deficiency due to low concentrations of the steroid precursors.

The decision to start substitution therapy with glucocorticoids to treat cortisol deficiency is based on cortisol concentrations after an ACTH stimulation test to simulate a severe stress-situation. Originally, to establish a threshold of a normal stress response, cortisol concentrations of patients that underwent major surgery (a severe stress-situation) and survived were used. These concentrations were compared to cortisol concentrations after insulin-induced hypoglycemia (19). Although cortisol concentrations after insulin-induced hypoglycemia were lower compared to a major surgery, the insulin hypoglycemia test provided the most acceptable means of assessing cortisol concentrations in response to stress (19). However, as many clinicians were reluctant or unable to use the insulin-induced hypoglycemia test, implementation of the ACTH stimulation test was considered. To test the applicability of the ACTH stimulation test to diagnose cortisol deficiency, cortisol concentrations

obtained with the insulin-induced hypoglycemia test were compared to ACTH stimulated cortisol concentrations (20). A good correlation was found between both tests and since then ACTH stimulation tests are performed to determine cortisol deficiency. A threshold of > 500 nmol/L for adequate adrenal function (reviewed in (21)) based on a fluorimetrical technique, which measures cortisol and corticosterone, but also small amounts of unknown substances (19), is still used. Current cortisol detection methods (LC-MS/MS) have improved significantly and new assay-specific lower reference limits of cortisol response to ACTH in healthy volunteers have been established (22). However, a new diagnostic cut-off value to determine cortisol deficiency has not been set yet.

Based on our findings, we believe that this cut-off value should include the glucocorticoid activity of adrenal steroid precursors. Especially when it concerns CAH patients as they have increased concentrations of these precursors. We propose that future studies establishing a new threshold will include cortisol, 11-deoxycortisol, 11-deoxycorticosterone, 11-hydroxyprogesterone, 21-deoxycrotisol, 17-hydroxyprogesterone, and progesterone concentrations. Until now, measurement of these steroids requires LC-MS/MS, a very specific and accurate, but costly measurement method, which is not available in every laboratory. As there are no alternatives yet to measure these steroids accurately, regional central facilities to measure these steroids are recommended.

A decision aid using the newly established threshold for cortisol deficiency should be developed to offer the patient personalized care by establishing a patient-specific glucocorticoid status value. The patient's total glucocorticoid activity might be determined using the formula below.

Total glucocorticoid activity within a patient = a ∗ [cortisol] + b ∗ [11-deoxycortisol] + c ∗ [11-deoxycorticosterone] + d ∗ [11-hydroxyprogesterone] + e ∗ [21-deoxycortisol] + f ∗ 17-hydroxyprogesterone] + g ∗ [progesterone]

This formula illustrates that the total glucocorticoid activity within a patient is the sum of the concentration of each steroid multiplied by a certain factor. Further research is needed to establish the numerical value of these factors, which will be difficult as many processes are involved in receptor activation and its biological response. Researchers should at least take into account the efficacy and potency of each steroid precursor. The efficacy of a steroid is reflected by the maximum effect (receptor activation) that can be reached and depends on receptor occupancy and the ability of the receptor complex to initiate a molecular response. The potency of a steroid is determined by the amount of steroid that is needed to produce a given effect, for which the EC_{50}, the concentration that causes 50% of the maximal effect, is often used. The potency of a

steroid depends on the affinity of the steroid to the receptor and the number of receptors available. After the development of the patient-specific glucocorticoid status value tool, guidelines for glucocorticoid treatment in CAH patients should be adapted to the newly established threshold of cortisol deficiency. Treatment might then be provided only to the patients with insufficient total glucocorticoid activity instead of treating patients based on deficient cortisol concentrations. Possibly, non-classic CAH patients would benefit most from this newly adapted threshold including all steroids with glucocorticoid activity as they only have mildly impaired cortisol concentrations and increased concentrations of steroid precursors. Currently, not all non-classic patients are treated with stress dosing of glucocorticoids even when their cortisol response is impaired. These patients can therefore help to establish the clinical validity of the patient-specific glucocorticoid status value tool.

Impaired gonadal function in male CAH patients

Although some untreated patients apparently are protected from adrenal crisis by the accumulation of precursor steroids with glucocorticoid activity (see above), in the past CAH patients had a substantial mortality risk due to the lack of effective treatment options and the subsequent occurrence of adrenal or salt-wasting crises. Since glucocorticoid and -if necessary- mineralocorticoid supplementation became available, CAH patients have a prolonged lifespan and, thus, long-term consequences of CAH became of more importance. One of the most important long-term complication in male CAH patients is the impairment of fertility and gonadal function. Literature so far reported contradicting results as fertility and gonadal function in male CAH patients were reported ranging from normal to severely impaired (23-30). In **chapter 7** we showed that gonadal dysfunction is a common complication in male CAH patients. Impaired fertility and gonadal function can have several causes: hypogonadotropic hypogonadism, testicular adrenal rest tumors, Sertoli cell dysfunction, impaired semen quality and psychosexual factors.

Hypogonadotropic hypogonadism

Testicular dysfunction caused by hypogonadotrophic hypogonadism is characterized by low testosterone concentrations combined with decreased gonadotropin concentrations in serum. However, in adult male CAH patients the use of testosterone concentrations in serum to diagnose gonadal dysfunction can be misleading, as measured testosterone in serum can be from adrenal and testicular origin. When the testosterone is derived from the testis, not much androstenedione is produced (31). In contrast, untreated or poorly controlled CAH patients have increased concentrations of

androstenedione (1). Therefore, it has been suggested by others (31) to use the ratio of androstenedione and testosterone concentrations (AD/T ratio) to determine the origin of measured total testosterone concentrations. A ratio higher than 0.5 indicates that a significant fraction of testosterone originates from adrenal steroidogenesis, and a ratio higher than 1 indicates that the majority of testosterone originates from adrenal steroidogenesis (31). In **chapter 7** we found that endocrine disturbances of the hypothalamus-pituitary-gonadal (HPG) axis (low gonadotropins) were more often present in patients with decreased testosterone concentrations compared to patients with normal testosterone concentrations. We also showed that patients where the majority of testosterone originates from the adrenal gland (AD/T ratio>1) had more often low gonadotropins compared to patients with testosterone of testicular origin. This indicates that adrenal androgens contribute to the suppression of gonadotropin production. Probably, adrenal androgens are aromatized to estrogens (mainly in glandular tissues), leading to increased estrogens concentrations, which give negative feedback on the HPG axis. Although other precursor steroids, such as 11-oxygenated androgens, are also suggested to contribute to the suppression of the HPG axis (32, 33), their exact role is not determined yet. We therefore recommend to include the AD/T ratio in combination with gonadotropin concentrations in the follow-up of adult male CAH patients to evaluate testicular function.

Testicular adrenal rest tumors

One of the most common complication in male CAH patients that can cause impaired gonadal function is the development of testicular adrenal rest tumor (TART)s. In **chapter 4** we present a review on TART. The overall reported prevalence in classic 21-hydroxylase deficient patients is 40% (range 14-89%), and the prevalence of TART in our European cohort described in **chapter 7** was 49%. TART occurs both in pediatric and in adult CAH patients, and an increase in prevalence is seen during puberty (34-38). TARTs are benign lesions of the testes that are typically located within the rete testis and can cause impaired gonadal function by obstruction of the seminiferous tubules or due to paracrine effects of hormones produced by TART.

Until now the origin and cause of TART remain uncertain. As reviewed in **chapter 4** TARTs have adrenal and testicular characteristics, therefore a pluripotent steroidogenic cell type is hypothesized to be the origin of TART. As the gonads and adrenal glands have the same primordial origin, cells from the adrenogonadal primordium are a possible candidate. GATA transcription factors are involved in adrenogonadal development (39) and recently a possible relation of GATA transcriptions factors with TART development in mice was described (40-42). In **chapter 5** we therefore determined GATA expression in human TART tissue and found gene expression of

GATA3, GATA4, and *GATA6*. We also found *GATA3* and *GATA6* expression in fetal and adult adrenal tissue, while *GATA4* was expressed in fetal and adult testicular tissue. This confirmed adrenal and testicular characteristics of TART and suggested that dysregulation of GATA transcription factors might be involved in TART development.

TARTs share morphological similarities with steroid-producing testicular Leydig cells. It is therefore difficult to histologically discriminate TART from Leydig cell tumors (LCTs) and several cases of LCTs in CAH patients have been described (43-48). Different treatment strategies are required as TARTs will only be surgically removed if severe pain complaints are present (49) whereas LCTs will be surgically removed with a testis-sparing surgery or orchiectomy (50). Few characteristics, such as bilateralism, diagnosis of CAH, shrinkage of tumor after intensified glucocorticoid treatment, and/or presence of Reinke crystals, are used to discriminate between TART and LCT, but none of them is completely accurate, resulting in a chance of misdiagnosis and consequently incorrect treatment, which is reported in at least 2 cases (51, 52). In chapter 5 we describe the use of GATA transcription factors as discriminative markers. High gene expression of *GATA3* and *GATA6* in TARTs was discriminative from low gene expression in LCTs. However, protein expression was also determined to improve the usefulness of these markers in the clinic, but immunohistochemical quantification of these GATA factors could not discriminate between TART and LCT. In chapter 4 several other characteristics are discussed, but until now no single marker is able to discriminate TART from LCT. Future research is necessary to identify a single marker that is able to discriminate between both pathologies or might combine several individual markers that combined can discriminate TART from LCT accurately to prevent misdiagnosis and incorrect treatment. Further studies should also investigate the use of these markers in less-invasive material, such as blood or urine, to be of applicable use. For now, clinicians should at least consider the presence of CAH, bilateralism, and possibly try intensified glucocorticoid treatment to discriminate TART from LCT.

Poor hormonal control, characterized by high ACTH, 17-hydroxyprogesterone, and androstenedione concentrations, seems to be associated with the development of TART (reviewed in chapter 4) as TART prevalence is higher in CAH patients that are more severely affected and thus have higher ACTH concentrations. In chapter 6 we describe a unique patient with recurrent Cushing disease after bilateral adrenalectomy, who developed testicular tumors. These tumors were similar to TART based on messenger RNA expression analysis and steroid metabolome profiling. This unique case also suggests the importance of chronic exposure to high concentrations of ACTH in TART development as this patient experienced high ACTH concentrations from 11 years old on. These ACTH concentrations ranged from 10 times to more than 200 times higher than normal. However, in chapter 7 and chapter 4 we describe TART in less

severely affected CAH patients, who have only mildly increased ACTH concentrations. This might imply that the duration and timing of increased ACTH concentrations is of more importance compared to the actual amount of ACTH present for the development of TART. Also other growth-promoting factors might be of importance, such as luteinizing hormone and angiotensin II, but this has yet to be studied in detail. ACTH binds to its receptor (MC2R), which signals via cyclic adenosine monophosphate (cAMP), a second messenger that can bind to cAMP response elements (CREB). One or several CREB sites are present in GATA genes and we showed in **chapter 5** that incubation of a fetal testis cell line with cAMP led to increased expression of *GATA3, GATA4,* and *GATA6*. This is line with previous studies that published increased expression of GATA transcription factors in gonadal cell lines after stimulation with cAMP (53-55). However, we could not determine whether ACTH influences *GATA* expression in TART as currently no ACTH-sensitive animal or cell model is available.

Currently no treatment exists yet to prevent the formation or treat TART. Intensified glucocorticoid treatment might cause shrinkage of TART in some, but definitely not in all CAH patients with TART (24, 56-61). Surgery is only recommended when severe pain complaints are present, as removal of the TART does not improve fertility outcomes (49). With no suitable therapy in place CAH patients should be informed about the risks of the development of TART and consequently the risk on infertility. We advise clinicians to monitor their CAH patients regularly with ultrasound, starting already in childhood, and offer CAH patients with TART to cryopreserve their semen for later fertility purposes. Furthermore, prevention of the development of TART should be pursued by optimizing treatment strategies already in childhood to pursue normal ACTH concentrations.

Quality of life in male patients with CAH

Several factors, such as having a chronic disease and experiencing long-term complications, can affect quality of life (QoL). Impaired QoL has been reported in CAH patients, although most studies focus on female patients as they experience genital virilization and masculinization and consequently often need corrective surgery (reviewed in Reisch *et al.* (62)). QoL studies in male CAH patients are scarce and the results contradicting showing either impaired (27, 63, 64), equal (65) or better (66) QoL in male CAH patients compared to reference populations. In **chapter 8** we showed that QoL in European male CAH patients is good, reporting scores similar to healthy reference populations. Male CAH patients even rated their QoL higher compared to chronically ill reference populations and compared to patients with a

disorder/difference of sex development (DSD), including Turner Syndrome, Klinefelter syndrome, XY female DSD, and XY male DSD. One of the differences between CAH and DSD is the presence of increased adrenal steroid precursors. These steroid precursors are able to activate the GR as described in chapter 2 and 3 of this thesis, which might explain why these patients experience less complaints of their cortisol deficiency than expected. Fewer complications might lead to better QoL observed in male CAH patients. Furthermore, we found in chapter 8 that QoL was higher in CAH males compared to CAH females and that undertreatment based on subjective scoring or increased 17-hydroxyprogesterone concentrations in male CAH patients was associated with higher QoL scores. This suggests that undertreatment resulting in androgen excess in male CAH patients does not impair QoL, in contrast to female CAH patients that suffer from virilization and masculinization due to androgen excess and have lower QoL. QoL was significantly higher in undertreated male CAH patients compared to overtreated male CAH patients, which might imply that male CAH patients suffer more form glucocorticoid excess due to overtreatment. Only based on QoL, one might suggest to undertreat these patient. However, untreated CAH patients have increased ACTH concentrations, which may lead to an increased risk of TART development and consequently decrease QoL. Treatment in CAH patients remains difficult and all advantages and disadvantages should be evaluated per patient to provide optimal treatment reducing the risk on long-term complications in CAH patients and increasing QoL (Fig. 9.2).

Concluding remarks

In this thesis, I describe the disturbed adrenal steroidogenesis and long-term complications regarding male gonadal function in CAH patients. We show that elevated adrenal steroid precursors can activate the GR and therefore are able to compensate for the cortisol deficiency, thereby possibly preventing the development of adrenal crises in some (untreated) CAH patients. Future research should focus on establishing a new threshold to determine cortisol deficiency based on all GR-activating steroids. In this thesis, we also show that impairment of gonadal function and fertility is a common complication in male CAH patients, although they rate their QoL as good. TART is one of the commonest complication in male CAH patients with a prevalence of 40% and occurs in children and adults with an increase in prevalence during puberty. TARTs are hyperplastic steroidogenic cells with adrenal and testicular characteristics.

Although the origin of TART is currently unknown, it is hypothesized that TART cells originate from a pluripotent cell, such as fetal Leydig cells or cells from the adrenogonadal primordium. Poor hormonal control, characterized by increased ACTH

concentrations, is associated with increased prevalence of TART, probably causing the hyperplasia of the pluripotent cells. Unfortunately, there are no treatment options yet to prevent TART development and no guidelines on how to treat TART are established. Future research should therefore focus on the development of TART to find druggable targets, possibly starting by developing more efficient therapies to lower ACTH concentrations. We recommend clinicians to increase male CAH patients' awareness of the risk of developing TART and consequently the risk on infertility. Patients should be screened regularly with ultrasound and semen preservation should be offered for future fertility purposes.

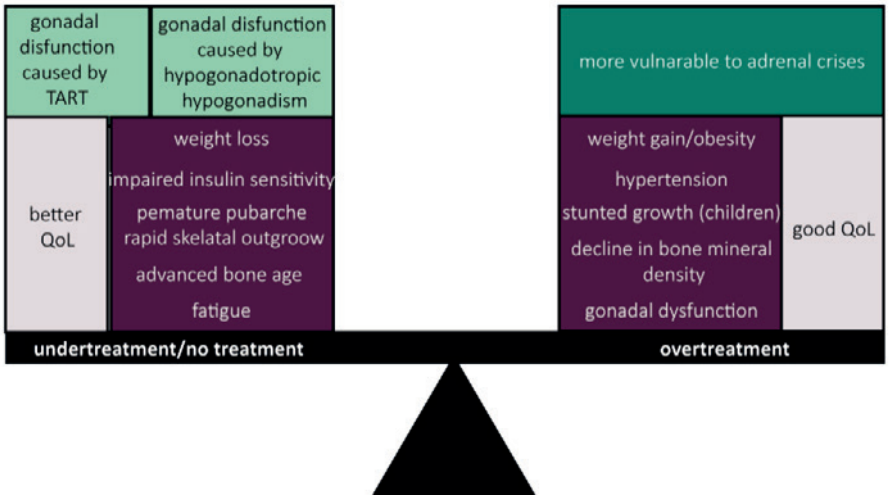

Fig. 9.2: The balance of glucocorticoid treatment within male congenital adrenal hyperplasia patients. Accurate treatment of the congenital adrenal hyperplasia (CAH) patient defined as normalization of elevated adrenal androgens with substitution of deficient steroids results in normalization of growth and normal development. However, it is hard to achieve accurate treatment as there is delicate balance between under-, accurate, and overtreatment. It is known that undertreatment or no treatment can lead to weight loss (2), impaired insulin sensitivity (67), premature pubarche and rapid skeletal outgrow (2), advanced bone age (2) and fatigue (2). **In this thesis**, we showed that in undertreated/untreated gonadal function can be impaired due to hypogonadotropic hypogonadism (**chapter 7**) caused by the aromatization of elevated adrenal androgens. Gonadal dysfunction can also be caused by testicular adrenal rest tumors (TARTs) (**chapter 4-7**). Undertreated CAH patients have increased ACTH concentrations, which is an important stimulating factor in the development of TART. Furthermore, we showed that quality of life (QoL), rated on a 0-100 scale (higher scores reflect better QoL), was significantly higher in undertreated CAH patients (QoL=70.8-100) compared to overtreated CAH patients (QoL=58.3-87.5)(**chapter 8**). Overtreated patients might be more vulnerable to adrenal crises as adrenal steroid precursors are decreased. Adrenal steroid precursors have glucocorticoid activity and might compensate for the cortisol deficiency (**chapter 2-3**). It was already known that overtreatment can lead to weight gain (2, 67), hypertension (67), stunted growth in children (2, 67) and give a decline in bone mineral density (2, 67). Also glucocorticoid induced suppression of the hypothalamus-pituitary-gonadal axis leading to hypogonadotropic hypogonadism was reported (26). All these advantages and disadvantages of over- or undertreatment should be evaluated per patient to provide optimal treatment. Abbreviations: CAH, congenital adrenal hyperplasia; QoL, quality of life.

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Nederlandse samenvatting

De bijnieren hebben belangrijke endocriene functies in ons lichaam. Zij zijn verantwoordelijk voor de productie van steroïdhormonen en catecholamines en regelen hiermee levensnoodzakelijke functies zoals de stressrespons of zoutregulatie. De bijnierschors produceert drie soorten steroïdhormonen: mineralocorticoïden (zouthormoon), glucocorticoïden (stresshormoon) en androgenen (mannelijke hormonen). Mineralocorticoïden regelen onze bloeddruk en elektrolytenbalans, glucocorticoïden (o.a. cortisol) regelen ons glucose metabolisme en immuunrespons en de androgenen geproduceerd door de bijnierschors leiden vooral bij vrouwen tot de ontwikkeling van pubisbeharing. De productie van deze hormonen is streng gereguleerd door het renine-angiotensine-aldosteronsysteem (regulatie van de mineralocorticoïden) en het hypofysair geproduceerd adrenocorticotroop hormoon (ACTH, regulatie van cortisol).

Het adrenogenitaal syndroom (AGS) is een groep zeldzame, overerfbare afwijkingen van de bijnierschors. AGS kenmerkt zich door verlaagde cortisol productie, met als gevolg verhoogde ACTH productie dat uiteindelijk leidt tot overstimulatie en hyperplasie van de bijnierschors. AGS wordt grotendeels veroorzaakt door een enzymatisch defect van 21-hydroxylase (CYP21A2 mutaties). In dit proefschrift concentreren wij ons met name op 21-hydroxylase deficiënte (21OHD) patiënten. Door de verhoogde ACTH productie accumuleren voorloperstoffen van de steroïdhormonen: de steroïd precursors. Deze worden voor een groot gedeelte omgezet in androgenen, aangezien deze productie niet-aangedaan is. AGS patiënten hebben dus een verstoorde hormoonbalans bestaande uit verhoogde androgeen en verlaagde cortisol en aldosteron concentraties. Ook lopen zij risico op het ontwikkelen van een bijniercrisis en het ontwikkelen van langetermijncomplicaties zoals verminderde gonadale functie. Dit proefschrift heeft als doel om meer inzicht te krijgen in de verstoorde bijnier steroïdogenese in AGS patiënten en de langetermijnconsequenties van AGS.

De rol van bijnier steroïd precursors in adrenogenitaal syndroom

Cortisoldeficiëntie kan leiden tot verschillende klachten, zoals zwakheid, vermoeidheid en gewichtsverlies, maar ook tot levensbedreigende bijniercrisissen met name in situaties van ziekte met grotere cortisolbehoefte. AGS patiënten maken te weinig cortisol en worden levenslang behandeld met glucocorticoïden. Ook ontvangen zij instructies om de dosering glucocorticoïden op te hogen bij ernstige stressmomenten zoals een operatie of ziekte. Sommige 21OHD patiënten rapporteren minder klinische tekenen van cortisoldeficiëntie dan verwacht wordt op basis van de mate van de

enzymdeficiëntie. In hoofdstuk 3 beschrijven we een cohort van langdurig onbehandelde AGS patiënten en biochemisch bevestigde cortisoldeficiëntie. Hoewel ongeveer de helft van de patiënten periodes van ernstige stress hebben doorgemaakt, heeft geen van hen tekenen van een bijniercrisis gerapporteerd. Dit toont aan dat in AGS patiënten de verwachte klinische klachten niet altijd overeenkomen met de biochemische diagnose van cortisoldeficiëntie in AGS patiënten

In hoofdstuk 2 en 3 van dit proefschrift stellen we dat de glucocorticoïde activiteit van de verhoogde concentraties van steroïd precursors voor cortisol en aldosteron in AGS patiënten mogelijk het gebrek aan klinische symptomen van cortisoldeficiëntie kan verklaren. Mineralocorticoïden en glucocorticoïden voeren hun functie uit door te binden aan de bijbehorende receptoren: de mineralocorticoïden receptor (MR) en de glucocorticoïden receptor (GR). Het steroïdhormoon bindt zich aan de receptor in het cytoplasma van de cel, waarna het receptor-steroïd complex zich verplaatst naar de kern van de cel (translocatie). Daar kan het receptorcomplex gentranscriptie initiëren (transactivatie) door te binden aan het hormoon-respons-element in de promotor regio van het desbetreffende gen. Kruisreactiviteit van steroïden is een bekend fenomeen en kan verklaard worden door de grote overlap in de structuurformule van de steroïden en in het DNA-bindende deel van de GR en MR. In hoofdstuk 2 hebben we beschreven dat 21-deoxycortisol kan binden aan de GR en dat het zowel translocatie als ook transactivatie veroorzaakt met eenzelfde potentie als cortisol. 17-hydroxyprogesteron en progesteron veroorzaken ook transactivatie via de GR, maar met een lagere potentie vergeleken met cortisol, terwijl androsteendion niet in staat is te binden en geen translocatie en transactivatie kan veroorzaken. In hoofdstuk 3 hebben we de potentie van 21-deoxycortisol, 17-hydroxyprogesteron en progesteron om via de GR transactivatie te veroorzaken bevestigd en hebben we de potentie van andere mineralocorticoïde en glucocorticoïde precursors bepaald. Deze resultaten komen overeen met enkele andere studies die de potentie voor GR transactivatie van slechts een beperkt aantal steroïden hebben bepaald. Uit de structuur-activiteit-relatie van de steroïd precursors bleek dat 3β-hydroxysteroïd dehydrogenatie een vereiste is voor een steroïd om transactivatie via de GR te kunnen veroorzaken. Verder vonden we ook dat de meest potente steroïden 11-gehydroxyleerd waren.

AGS patiënten hebben een hormonale disbalans met lage cortisol en aldosteron concentraties, hoge androgeen concentraties en accumulatie van de steroïd precursors. 17-hydroxyprogesteron concentraties in onbehandelde AGS patiënten kunnen tot wel meer dan 100x verhoogd zijn vergeleken met de normale referentiewaarden en worden daarom ook gebruikt voor de diagnose van AGS. In hoofdstuk 3 hebben we inderdaad verhoogde mediaan concentraties van diverse steroïden gemeten in serum van 21OHD patiënten vergeleken met controles: 21-

deoxycortisol (alleen meetbaar in 21OHD patiënten), 17-hydroxyprogesteron (127x verhoogd) en progesteron (29x verhoogd). In 11-hydroxylase deficiënte patiënten vonden we hogere concentraties van 11-deoxycortisol (457x hoger) en 11-deoxycorticosteron (55x hoger) in vergelijking met controles. Deze resultaten laten zien dat steroïd precursors accumuleren. Al deze steroïden zijn in staat transactivatie te veroorzaken via de GR met vergelijkbare of met lagere potentie in vergelijk met cortisol. Daarom zouden deze steroïden mogelijk kunnen compenseren voor de cortisoldeficiëntie.

Momenteel worden AGS patiënten behandeld met glucocorticoïden als substitutie voor cortisol. Dit verlaagt ook de ACTH concentraties, wat weer het gehalte van androgenen afkomstig uit de bijnier verlaagt. Turcu *et al.* hebben laten zien dat 17-hydroxyprogesteron, progesteron en 21-deoxycortisol concentraties in behandelde 21OHD patiënten nog steeds hoger waren vergeleken met gezonde controles. Een vergelijking van deze concentraties in behandelde 21OHD patiënten met de concentraties van ons cohort van onbehandelde 21OHD patiënten (beschreven in hoofdstuk 3) laat zien dat de concentraties van 17-hydroxyprogesteron, progesteron en 21-deoxycortisol lager waren in behandelde 21OHD patiënten. Dit geeft aan dat behandeling met glucocorticoïden inderdaad de concentraties van de steroïd precursors verlaagt en suggereert dat het compensatiemechanisme van deze steroïden verlaagd zal zijn door behandeling met glucocorticoïden. Daarom is het belangrijk dat patiënten uitvoerig geïnformeerd worden over het risico op bijniercrisissen en het belang van therapietrouw en stressdoseringen. Echter, in ontwikkelingslanden, waar medicatie niet altijd beschikbaar is en ook kennis en infrastructuur niet optimaal zijn, zal het plotseling stoppen van glucocorticoïden het risico op een bijniercrisis mogelijk vergroten, aangezien zowel cortisol als steroïd precursors concentraties laag zijn door behandeling.

Het besluit om te starten met substitutietherapie met glucocorticoïden om cortisoldeficiëntie te behandelen is gebaseerd op cortisol concentraties na een ACTH stimulatietest. Een afkapwaarde van >500 nmol/L voor adequate bijnier functie wordt nog steeds gebruikt, terwijl deze waarde gebaseerd is op een fluorimetrische bepaling die cortisol en corticosteron meet maar ook kleine hoeveelheden onbekende stoffen. De huidige methoden om cortisol te detecteren (vloeistofchromotografie met tandem massaspectometer (LC-MS/MS)) zijn aanzienlijk beter en nieuwe lagere assayspecifieke referentiegrenzen voor de cortisolrespons op ACTH bij gezonde vrijwilligers zijn vastgesteld. Een nieuwe diagnostische afkapwaarde om cortisoldeficiëntie te diagnosticeren is echter nog niet vastgesteld. Op basis van onze bevindingen raden we aan alle steroïd precursors met glucocorticoïde activiteit in deze diagnostische afkapwaarde mee te nemen. Zeker in het geval van AGS patiënten, waar verhoogde

concentraties van deze precursors aanwezig zijn. We stellen voor dat toekomstige studies die een afkapwaarde gaan vaststellen rekening houden met cortisol, 11-deoxycortisol, 11-deoxycorticosteron, 11-hydroxyprogesteron, 21-deoxycortisol, 17-hydroxyprogesteron en progesteron concentraties. Tot nu toe vereist het meten van deze steroïden LC-MS/MS, een zeer specifieke en nauwkeurige, maar tevens kostbare meetmethode, die nog niet in elk laboratorium beschikbaar is. Vandaar dat wij adviseren om deze steroïden in regionale expertisecentra te meten.

Verminderde gonadale functie bij mannelijke AGS patiënten

Sinds de beschikbaarheid van substitutietherapie met glucocorticoïden en mineralocorticoïden en betere monitoring bereiken AGS patiënten de volwassen leeftijd zonder ernstige complicaties en worden de langetermijngevolgen van AGS duidelijker. In hoofdstuk 7 beschrijven wij dat verminderde gonadale disfunctie een van de belangrijkste langetermijncomplicaties is bij mannelijke AGS patiënten. Verminderde vruchtbaarheid door gonadale disfunctie kan verschillende oorzaken hebben: hypogonadotroop hypogonadisme, testiculaire bijnier resttumoren, Sertolicel disfunctie en verminderde zaadkwaliteit.

Hypogonadotroop hypogonadisme

Testiculaire disfunctie veroorzaakt door hypogonadotroop hypogonadisme wordt over het algemeen gedefinieerd als lage testosteron concentraties in combinatie met lage gonadotropine concentraties in serum. Slecht ingestelde AGS patiënten hebben vaak lage gonadotropines door het remmende effect van bijnierandrogenen, maar in tegenstelling tot andere condities wel normale testosteron concentraties. Dit testosteron is omgezet uit een verhoogde productie van bijnierandrogenen (androsteendion). Daarom is door anderen gesuggereerd om de verhouding tussen androsteendion en testosteron (AD/T ratio) te gebruiken om de oorspong van de gemeten totale testosteron concentraties te bepalen. Een ratio hoger dan 0,5 geeft aan dat een significante fractie van testosteron afkomstig is uit de bijnier en een ratio hoger dan 1 geeft aan dat de meerderheid van testosteron afkomstig is van de bijnier. In hoofdstuk 7 wordt beschreven dat patiënten met een AD/T ratio>1 vaker lage gonadotropines hadden in vergelijking met patiënten met een ratio <0,5. Dit suggereert dat bijnier androgenen bijdragen aan de onderdrukking van de productie van gonadotropines. Waarschijnlijk worden deze androgenen voornamelijk in klierweefsel gearomatiseerd tot oestrogenen, welke leiden tot negatieve feedback op de hypothalamus-hypofyse-gonade-as. We adviseren om bij slecht ingestelde AGS

patiënten de AD/T ratio in combinatie met gonadotropines te meten voor de evaluatie van de gonadale functie.

Testiculaire bijnier resttumoren

Een van de meest voorkomende complicaties bij mannelijke AGS patiënten is de ontwikkeling van testiculaire bijnier resttumoren (beter bekend onder de Engelstalige afkorting TARTs: "testicular adrenal rest tumors"). In **hoofdstuk 4** presenteren we een overzichtsartikel over TART, waarin de totaal gerapporteerde prevalentie bij 21OHD patiënten 40% is (variërend van 14-89%). De prevalentie van TART in ons Europese cohort (**hoofdstuk 7**) was 49%. TART komt zowel voor bij AGS kinderen en volwassen, waarbij een toename in prevalentie wordt gezien gedurende de puberteit. TARTs zijn goedaardige gezwellen die zich binnen de rete testis bevinden en kunnen leiden tot gonadale disfunctie door obstructie van de zaadbuisjes of door paracriene effecten van hormonen geproduceerd door TART.

Tot nu toe is de etiologie van TART niet duidelijk. Omdat TART zowel bijnier als testiculaire eigenschappen heeft (**hoofdstuk 4**) veronderstellen wij dat TART ontstaat uit pluripotente steroïdogene cellen. Aangezien de bijnier en gonaden embryologisch eenzelfde oorsprong hebben zijn cellen uit het adrenogonadaal primordium een mogelijke optie als oorsprong voor TART. GATA-transcriptiefactoren zijn betrokken bij de adrenogonadale ontwikkeling en onlangs werd een mogelijke relatie van GATA-transcriptiefactoren met TART-ontwikkeling in muizen beschreven. In **hoofdstuk 5** hebben we daarom de GATA-expressie in menselijk TART-weefsel bepaald en genexpressie van *GATA3*, *GATA4* en *GATA6* gevonden. Ook vonden we *GATA3* en *GATA6* expressie in foetaal en volwassen bijnierweefsel en *GATA4* expressie in foetaal en volwassen testisweefsel. Dit bevestigde de bijnier- en testiculaire eigenschappen van TART en suggereerde dat ontregeling van GATA-transcriptiefactoren mogelijk betrokken zouden kunnen zijn bij TART-ontwikkeling.

TARTs delen morfologische gelijkenissen met steroïd-producerende testiculaire Leydigcellen. Het is daarom moeilijk om TART histologisch van Leydigcel tumoren (LCTs) te onderscheiden en er zijn verschillende casussen van LCTs bij AGS patiënten bekend. Voor de behandeling van TARTs en LCTs zijn verschillende strategieën nodig, aangezien TARTs alleen chirurgisch worden verwijderd als er ernstige pijnklachten optreden, terwijl LCTs chirurgisch worden verwijderd met een testissparende operatie of orchiëctomie. Slechts enkele kenmerken, waaronder bilateralisme, diagnose van AGS, krimp van de tumor na behandeling met glucocorticoïden en/of aanwezigheid van Reinke kristallen kunnen worden gebruikt om onderscheid te maken tussen TART en LCT, maar geen van alle is volledig onderscheidend. Dit kan leiden tot een verkeerde diagnose en onjuiste behandeling die in ten minste twee gevallen is gerapporteerd. In

hoofdstuk 5 beschrijven we het gebruik van GATA-transcriptiefactoren als onderscheidende markers. *GATA3* en *GATA6* komen hoog tot expressie in TARTs, terwijl deze laag tot expressie komen in LCTs. Om de bruikbaarheid van deze markers in de kliniek te verbeteren is ook de eiwitexpressie bepaald, maar immunohistochemische kwantificering van deze GATA-transcriptiefactoren kon helaas geen onderscheid maken tussen TART en LCT. In hoofdstuk 4 worden verschillende andere kenmerken besproken, maar tot nu toe kan geen enkele marker het onderscheid maken tussen TART en LCT. Toekomstig onderzoek is nodig om een marker of combinatie van verschillende markers te identificeren die in staat is om onderscheid te maken tussen beide pathologieën wat tot een juiste diagnose en behandeling leidt. Verder onderzoek zou zich moeten richten op het identificeren van markers in minder invasief te verkrijgen materialen zoals bloed of urine.

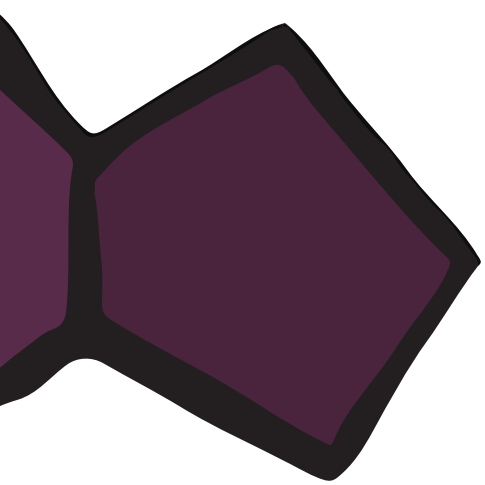
Slechte hormonale controle, gekenmerkt door hoge ACTH, 17-hydroxyprogesteron en androsteendion concentraties, wordt geassocieerd met de ontwikkeling van TART (beschreven in hoofdstuk 4), aangezien de prevalentie van TART hoger is in de meest aangedane AGS patiënten met hogere ACTH concentraties. In hoofdstuk 6 beschrijven we een unieke patiënt met terugkerende Cushing na bilaterale adrenalectomie die testiculaire tumoren ontwikkelde. Deze tumoren waren vergelijkbaar met TART op basis van mRNA en steroïd expressie analyse. Deze casus suggereert ook het belang van chronische blootstelling aan hoge concentraties ACTH in TART-ontwikkeling, aangezien deze 27-jarige patiënt al aantoonbare hoge ACTH concentraties ondervond vanaf zijn 11^{e} levensjaar. Deze ACTH concentraties varieerden in de loop der tijd van 10 tot 200 keer hoger dan normaal. In hoofdstuk 7 en hoofdstuk 4 beschrijven we echter de aanwezigheid van TART in minder aangedane AGS patiënten, die slechts licht verhoogde ACTH concentraties hebben. Dit kan impliceren dat de duur en het tijdstip van verhoogde ACTH concentraties van groter belang zijn voor TART-ontwikkeling in vergelijking met de aanwezige hoeveelheid ACTH. Ook kunnen andere groeibevorderende factoren van belang zijn, zoals LH en angiotensine II, maar dit moet nog in detail worden bestudeerd. ACTH bindt zich aan de receptor (MC2R), die signalen afgeeft via cyclisch adenosine monofosfaat (cAMP), een secundaire boodschapper die kan binden aan cAMP-responselementen (CREB). CREB-sites zijn aanwezig in GATA-genen en in hoofdstuk 5 hebben we aangetoond dat incubatie van een foetale testiscellijn met cAMP leidde tot verhoogde expressie van *GATA3, GATA4* en *GATA6*. Dit komt overeen met eerdere studies die verhoogde expressie van GATA in gonadale cellijnen na stimulatie met cAMP aantoonden. We konden echter niet bepalen of ACTH de GATA-expressie in TART beïnvloedt, omdat er momenteel geen ACTH-gevoelig dier- of celmodel beschikbaar is.

Kwaliteit van leven in mannelijke AGS patiënten

Verschillende factoren, zoals het hebben van een chronische ziekte en het ervaren van langdurige complicaties, kunnen de kwaliteit van leven (KvL) beïnvloeden. Verminderde KvL is bekend bij AGS patiënten, waarbij de meeste onderzoeken zich richten op vrouwelijke patiënten omdat zij genitale virilisatie en masculinisatie ervaren en daarvoor vaak corrigerende chirurgie nodig hebben. KvL-onderzoeken bij mannelijke AGS patiënten zijn schaars en de gerapporteerde resultaten zijn in tegenspraak met elkaar aangezien zowel een verminderde, gelijke als betere KvL is gerapporteerd. In **hoofdstuk 8** hebben we aangetoond dat de KvL bij Europese mannelijke 21OHD patiënten goed is, waarbij de gerapporteerde scores vergelijkbaar zijn met gezonde referentiepopulaties. Mannelijke AGS patiënten beoordeelden hun KvL zelfs hoger in vergelijking met chronisch zieke referentiepopulaties en vergeleken met patiënten met een stoornis/verschil in geslachtsontwikkeling (DSD), waaronder het syndroom van Turner, Klinefelter-syndroom, XY-vrouwelijke DSD en XY-mannelijke DSD. Een van de verschillen tussen AGS en DSD is de verhoogde aanwezigheid van bijnier steroïd precursors. Deze precursors kunnen de GR activeren zoals beschreven in **hoofdstuk 2 en 3** van dit proefschrift, wat zou kunnen verklaren waarom deze patiënten minder klachten van hun cortisoldeficiëntie ervaren dan verwacht. Minder complicaties kunnen leiden tot een betere KvL, wat is waargenomen bij mannelijke AGS patiënten. Verder vonden we in **hoofdstuk 8** dat de KvL hoger was bij AGS mannen vergeleken met AGS vrouwen en dat onderbehandeling op basis van een subjectieve score of verhoogde 17-hydroxyprogesteron concentraties bij mannelijke AGS patiënten geassocieerd waren met hogere KvL-scores. Dit suggereert dat onderbehandeling resulterend in een androgeenoverschot bij mannelijke AGS patiënten de KvL niet nadelig beïnvloedt, in tegenstelling tot vrouwelijke AGS patiënten die lijden aan virilisatie en masculinisatie vanwege een overmaat aan androgeen (en een lagere KvL hebben). KvL was significant hoger bij onderbehandelde mannelijke AGS patiënten vergeleken met overbehandelde patiënten, wat zou kunnen inhouden dat deze mannen meer lijden van overmatige glucocorticoïden als gevolg van overbehandeling. On(der)behandelde AGS patiënten hebben daarentegen verhoogde ACTH concentraties, wat kan leiden tot een verhoogd risico op TART-ontwikkeling en daardoor een verminderde KvL. Behandeling bij AGS patiënten blijft moeilijk en alle voordelen en nadelen moeten per patiënt worden geëvalueerd om een optimale behandeling te bieden die het risico op langetermijncomplicaties vermindert en een goede KvL waarborgt.

Slotopmerkingen

In dit proefschrift beschrijf ik de verstoorde bijniersteroïdogenese en enkele langetermijncomplicaties met betrekking tot de mannelijke gonadale functie bij AGS patiënten. Wij laten zien dat verhoogde steroïd precursors de GR kunnen activeren, waardoor mogelijk de ontwikkeling van bijniercrisissen bij sommige (onbehandelde) AGS patiënten wordt voorkomen. Toekomstig onderzoek moet zich richten op de klinische betekenis van deze steroïden. In dit proefschrift laten we ook zien dat een verminderde werking van de testes, met name veroorzaak door TART, een veel voorkomende complicatie is bij mannelijke AGS patiënten. Helaas zijn er nog geen behandelingsopties om TART-ontwikkeling te voorkomen en er zijn geen richtlijnen vastgesteld voor de behandeling van TART. Toekomstig onderzoek moet zich daarom richten op de etiologie van TART en het vinden van een gerichte behandeling. Zonder geschikte therapie zouden AGS patiënten moeten worden geïnformeerd over de risico's van de ontwikkeling van TART en het bijbehorende risico op onvruchtbaarheid. Patiënten moeten regelmatig worden gescreend met echografie en preservatie van sperma moet worden aangeboden voor toekomstige vruchtbaarheidsdoeleinden. Ondanks serieuze langetermijncomplicaties rapporteren mannelijke AGS patiënten een goede kwaliteit van leven.



Appendices

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Acknowledgements - Dankwoord

List of abbreviations

0-9

11-hydroxylase	cytochrome P450 11B1
11β-HSD	corticosteroid 11β-dehydrogenase
11OHD	11-hydroxylase deficient
17β-HSD	testosterone or estradiol 17β-dehydrogenase
17-hydroxylase	17α-hydroxylase/17,20lyase
21OHD	21-hydroxylase deficient
3β-HSD	3β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$isomerase
5α-reductase	3-oxo-5α-steroid 4-dehydrogenase 1
95%CI	95% confidence interval

A

ACTH	adrenocorticotropic hormone
AD/T	androstenedione/testosterone
AMH	anti-Müllerian hormone
AUC	area under the curve

C

CAH	congenital adrenal hyperplasia
cAMP	cyclic adenosine monophosphate
COS-7	fibroblast-like cell line derived from monkey kidney tissue
CREB	cAMP response elements
CRH	corticotrophin-releasing hormone

D

DHEA(-S)	dehydroepiandrosterone (sulfate)
DSD	disorder/difference of sex development
DHT	dihydrotestosterone

E-F

EC_{50}	estimated concentration for 50% transactivation
FSH	follicle stimulating hormone

G

GFP	green fluorescent protein
GR	glucocorticoid receptor

H

H295RA	ACTH-sensitive adrenocortical cell line
hCG	human chorionic gonadotropin

HDDST	high-dose dexamethasone suppression test
HEK293	human embryonic kidney cells
HeLa	cell line derived from cervical cancer from Henrietta Lacks
HPA	hypothalamus-pituitary-adrenal
HPG	hypothalamus-pituitary-gonadal
hs181.tes	human fetal testis cell line

I

IC_{50}	concentration that reduces binding of the radioligand by half
IQR	interquartile range

L

LC-MS/MS	liquid chromatography-tandem mass spectrometry
LCTs	Leydig cell tumors
LH	luteinizing hormone
LRL	lower reference limit

M

MMTV-luc	firefly luciferase reporter construct
MR	mineralocorticoid receptor
mRNA	messenger RNA
MWU	Mann-Whitney U

O

OR	odds ratio

P

pRL-TK	renilla luciferase construct

Q

QoL	quality of life
qPCR	quantitative polymerase chain reaction

R

ROC	receiver operating characteristic

S

SHBG	sex hormone-binding globulin

T

TARTs	testicular adrenal rest tumors

List of publications

Parker BC, **Engels M**, Annala M, Zhang W. Emergence of FGFR family gene fusions as therapeutic targets in a wide spectrum of solid tumours. J Pathol. 2014 Jan;232(1):4-15.

Pijnenburg-Kleizen KJ, **Engels M**, Mooij CF, Griffin A, Krone N, Span PN, van Herwaarden AE, Sweep FC, Claahsen-van der Grinten HL. Adrenal Steroid Metabolites Accumulating in Congenital Adrenal Hyperplasia Lead to Transactivation of the Glucocorticoid Receptor. *Endocrinology.* 2015 Oct;156(10):3504-10. doi: 10.1210/en.2015-1087.

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Engels M, Gehrmann K, Falhammar H, Webb EA, Nordenström A, Sweep FC, Span PN, van Herwaarden AE, Rohayem J, Richter-Unruh A, Bouvattier C, Köhler B, Kortmann BB, Arlt W, Roeleveld N, Reisch N, Stikkelbroeck NMML, Claahsen-van der Grinten HL; dsd-LIFE group. Gonadal function in adult male patients with congenital adrenal hyperplasia. *Eur J Endocrinol.* 2018 Mar;178(3):285-294. doi: 10.1530/EJE-17-0862.

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Engels M*, Verhees MJM*, Span PN, Sweep FCGJ, van Herwaarden AE, Falhammar H, Nordenström A, Webb EA, Richter-Unruh A, Roeleveld N, Bouvattier C, Brac de la Perrière A, Arlt W, Reisch N, Köhler B, Rapp M, Stikkelbroeck NMML and Claahsen-van der Grinten HL on behalf of the dsd-LIFE group. Quality of life in adult males with congenital adrenal hyperplasia. *Submitted*. 2018.

Gehrmann K*, **Engels M***, Bennecke E, Bouvattier C, Falhammar H, Kreukels B, Nordenström A, Reisch N, Gehrmann N, Stikkelbroeck NMML, Quinkler M, Claahsen-van der Grinten HL, Köhler B, on behalf of the dsd-LIFE group. Sexuality of males with congenital adrenal hyperplasia. *In prep*. 2018.

* Authors contributed equally to this work

Curriculum Vitae

Manon Engels was born on 17 January 1991 in Mill en St. Hubert, the Netherlands. She graduated from high school at Merletcollege Cuijk in 2009. In 2012 she obtained her bachelor Biomedical Sciences at the Radboud University in Nijmegen. During her bachelor, she performed one internship at the department of Experimental Urology, Radboud university medical center where she investigated the improvement of tumor cell drug sensitivity by EMT-associated microRNAs in urological cancers. She then started her master in Biomedical Sciences with two majors at the Radboud University in Nijmegen. For her major in human pathobiology, Manon performed her internship in Houston, Texas at the pathology department of the MD Anderson Cancer Center under supervision of dr. Brittany Parker Kerrigan. She investigated the molecular mechanism of temozolomide resistance conferred by the FGFR3-TACC3 fusion gene in glioblastoma. For her second major in epidemiology, Manon performed her internship at the department of Experimental Urology in collaboration with the department for Health Evidence under supervision of prof. Bart Kiemeney and Dr. Gerald Verhaegh, where she investigated the diagnostic value of a miRNA panel for recurrence after primary treatment of non-muscle invasive bladder cancer. After obtaining her master in 2014, Manon joined the department of Pediatrics and the department of Laboratory Medicine in 2015 where she investigated the causes and consequences of congenital adrenal hyperplasia, supervised by dr. Claahsen-van der Grinten, prof. Sweep, and dr. Span. Her work resulted in publications in the highest-ranking journals in the endocrinology field (topic: adrenal), an approved grand application from the International Fund of Congenital Adrenal hyperplasia in 2016 and several (awarded) presentations in national and international conferences.

Research data management

Research data presented in this thesis and obtained during this PhD at the department of Pediatrics and department of Laboratory Medicine at the Radboud university medical center (Radboudumc) were archived according to the Findable, Accessible, Interoperable, and Reusable (FAIR) principles. The data was stored digitally at a local server, supported by the Information and Communications Technology (ICT) of Radboudumc and on paper in the form of labjournals. Data stored on the local servers were replicated daily to a server of the university.

Human studies in **chapter 3, 5, 6, 7 and 8** were conducted according to the principles accepted in the declaration of Helsinki. All participants gave oral and/or written consent to participate in the study. Additional measurements in coded samples were in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.federa.org/codes-conduct). The research was approved by the local ethical committee of the Diponegoro University **(chapter 3)**, by the institutional review board: CMO Radboudumc **(chapters 5 and 6)**, or by the medical ethics committee at the Charité Universitätsmedizin Berlin and the local ethics committees of the other study centers **(chapters 7 and 8)**. All data generated or analyzed in this thesis are included in published articles and its additional files are available from the associated corresponding author on request.

Institute for Molecular Life Sciences
Radboudumc

RIMLS Portfolio

Name PhD student:	*Manon Engels*	PhD perioid:	*15-01-2015 until 31-12-2018*
Departments:	*Pediatrics and Laboratory Medicine*	Promotors:	*prof. dr. C.G.J. Sweep* *prof. dr. C. Noordam*
Graduate school:	*Radboud Institute for Molecular Life Sciences*	Copromo-tors:	*dr. H.L. Claahsen-van der Grinten* *dr. P.N. Span*

TRAINING ACTIVITIES	Year(s)	ECTS
a) Courses & Workshops		
- Introduction day Radboudumc	2015	0.25
- RIMLS Graduate Course	2015	1.75
- Several workshops; how to write abstract / poster presentation / present science / search for grants	2016	0.5
- "Management voor Promovendi" course	2016	2
- Scientific Integrity	2016	1
- "Loopbaanmanagement" course	2016	0.8
- Scientific writing course	2016	3
- Advanced conversation course	2017	1.5
- Education in a Nutshell course	2017	1
- "Effectieve schrijfstrategieën" course	2017-2018	3
- Several career workshops	2018	0.6
- "Solliciteren en Netwerken" course	2018	0.8
b) Seminars & Lectures		
- RIMLS Radboud Research Rounds or Radboud Grand rounds or Science meets Business	2015-2018	6.1
- RIMLS seminars	2015-2018	3
- RIMLS Technical forums	2015-2018	0.7
c) (Inter)national Symposia & Congresses		
- RIMLS Radboud New Frontiers	2015-2016	1
- RIMLS PhD retreat; 3x#, 1x*	2015-2018	2.75
- Radboud Adrenal Center Meeting, 2x*	2015-16, 2018	0.7
- TML research meetings 1x*	2015-2018	2.25
- Amalia research meetings, 3x*	2015-2018	2.6
- Radboud Science Day#	2016	0.5
- Dutch Endocrine Meeting, 1x#, 3x*	2016-2018	2.5
- ESPE 2x#	2016, 2018	2.5
- ECE #	2017	1.5
- I-DSD*	2017	1.25
- Amalia Science Day*	2018	0.5
d) Other		
- review of scientific publication for Andrology	2016	0.1
- BCF career event	2017	0.25

- PON career day	2018	0.1
TEACHING ACTIVITIES	Year(s)	ECTS
e) Lecturing NA		
f) Other - Supervision of master student Myrthe Verhees	2017-2018	1
TOTAL		45.5

Oral and poster or laptop presentations are indicated with a * and # after the name of the activity, respectively.

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Op 15 januari 2015 begon ik mijn promotieonderzoek en nu bijna 4 jaar later kan ik op 18 december 2018 mijn proefschrift verdedigen. Twee bijzondere data om te onthouden. Het onderzoek dat in dit boekje beschreven staat is natuurlijk niet alleen door mij tot stand gekomen. Onderzoek staat voor mij per definitie gelijk aan samenwerken en van elkaars specialisme en kwaliteiten gebruik maken. Er hebben dan ook veel personen bijgedragen aan dit proefschrift die ik graag wil bedanken voor de bijdrage die ze geleverd hebben.

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al heb ik helaas niet mijn titel kunnen behouden. Annelies en Frans, bedankt dat jullie me op weg hebben geholpen in het lab en op de kweek. Ook nog een speciaal bedankje voor de promovendi van TML en KG, in het bijzonder Kioa, Anne, Pippa, Anke, Walinka en Sanne, zonder jullie waren het RIMLS-retreat, symposia en cursussen toch echt een stuk saaier geweest. Daarnaast ook een bedankje voor mijn kinderendocrinologie-collega's: de 'moord-tocht' in de binnenstad en het schilderuitje zal ik niet snel vergeten.

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